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Circulating tumor cells in personalized cancer therapy

Cirkulující nádorové buňky v personalizované onkologické terapii

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Klíčová slova: onkologické nemoci, nádorové biomarkery, metastazování, cirkulující nádorové buňky, personalizována medicína

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Content

List of abbreviations

ABC	Adenosine Triphosphate - Binding Cassette
ABCA	Adenosine Triphosphate - Binding Cassette Subfamily A
ABCB1	Adenosine Triphosphate - Binding Cassette Subfamily B Member 1
ABCC (MRP)	Adenosine Triphosphate - Binding Cassette Subfamily C
ABCG	Adenosine Triphosphate - Binding Cassette Subfamily G
AKT	Protein Kinase B
ALDH	Aldehyde Dehydrogenase
ALDH1	Aldehyde Dehydrogenase 1
AR	Androgen Receptor
ATM	Ataxia-Telangiesctasia mutated
ATP	Adenosine Triphosphate
BC	Breast Cancer
BMP	Bone Morphogenic Protein
BRCA	Breast Cancer
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
CA-125 (MUC16)	Cancer Antigen 125
CA 15-3	Cancer Antigen 125
CDH1	E-cadherin
CEA	Carcinoembryonic Antigen
CGH	Comparative Genome Hybridization
CHEK2	Checkpoint Kinase 2
CMCs	Circulating Melanoma Cells
CNHC	Circulating Non-Hematologic Cells
CNHC-BF	Circulating Non-Hematologic Cells with Benign Features
CNHC-MF	Circulating Non-Hematologic Cells with Malignant Features
CNHC-UMF	Circulating Non-Hematologic Cells with Uncertain Malignant Features
CNVs	Copy Number Variations
CRC	Colorectal Cancer
CSCs	Circulating Stem Cells
CT	Computed Tomography
ctDNA	Cell-Free (Circulating) Tumor Deoxyribonucleic Acid
CTC	Circulating Tumor Cells
CUPs	Cancers of Unknown Primary Origin
DAPI	4',6-Diamidino-2-Phenylindole
DFS	Disease-Free Survival
DNA	Deoxyribonucleic Acid
eBC	Early Breast Cancer
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor

EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cellular Adhesion Molecule
ERCC1	Excision Repair Cross-Complementation Group 1
ESR	Estrogen Receptor
ESR1	Estrogen Receptor Alpha
FGF	Fibroblast Growth Factor
FISH	Fluorescence *In Situ* Hybridization
HER2	Human Epidermal Growth Factor Receptor 2
HIF	Hypoxia-Inducible Factor
HR	Hormone Receptors
KRT8	Cytokeratin-8
KRT14	Cytokeratin-14
KRT18	Cytokeratin-18
KRT19	Cytokeratin-19
LB	Liquid Biopsy
MACS	Magnetic Cell Sorter
mBC	Metastatic Breast Cancer
MDR	Multidrug Resistance
MET	Mesenchymal-Epithelial Transition
MFS	Metastatic -Free Survival
MGB	Mammaglobin
MIB1	Mindbomb E3 Ubiquitin Protein Ligase 1
MMP	Matrix Metalloproteinase
MRP (ABCC)	Adenosine Triphosphate - Binding Cassette Subfamily C
MRP1 (ABCC1)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 1
MRP2 (ABCC2)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 2
MRP3 (ABCC3)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 3
MRP4 (ABCC4)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 4
MRP5 (ABCC5)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 5
MRP6 (ABCC6)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 6
MRP7 (ABCC10)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 10
MRP8 (ABCC11)	Adenosine Triphosphate - Binding Cassette Subfamily C Member 11
MRPs	Multidrug-Resistance Proteins
MUC4	Mucin 4
mRNA	Messenger Ribonucleic Acid
MYC	C-Myc
NACT	Neoadjuvant Chemotherapy
NBD	Nucleotide Binding Domain
NBDs	Nucleotide Binding Domains
NGS	Next Generation Sequencing
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival

p63	Transformation-Related Protein 63
PALB2	Partner And Localizer Of BRCA2
PARP	Poly-ADP Ribose Polymerase
PCR	Polymerase Chain Reaction
pCR	Pathological Complete Response
PD-L1	Programmed death-ligand 1
PDGF	Platelet Derived Growth Factor
PET	Positron Emission Tomography
PFS	Progression-Free Survival
PGR	Progesterone Receptor
PI3K	Phosphatidylinositol-3-Kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homolog
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RFS	Recurrence-Free Survival
RNA	Ribonucleic Acid
SCLC	Small Cell Lung Cancer
SCSs	Stem Cells Systems
Shh	Sonic Hedgehog
STK11	Serine/Threonine Kinase 11
TGF-β	Transforming Growth Factor β
TMD	Transmembrane Domain
TMDs	Transmembrane Domains
TNBC	Triple Negative Breast Cancer
TNM	Tumor, Nodes, Metastasis
VEGF	Vascular Endothelial Growth Factor
VEGF-C	Vascular Endothelial Growth Factor-C
VEGF-D	Vascular Endothelial Growth Factor-D
VEGFR	Vascular Endothelial Growth Factor Receptor
VEGFR-3	Vascular Endothelial Growth Factor Receptor-3
VIM	Vimentin
WHO	The World Health Organisation

1. Introduction

Cancer is known as one of the most frequent diseases that are responsible for morbidity and mortality worldwide. In general, cancer disease is characterized by uncontrolled growth of cells. Cancer cells proliferate in an uncontrolled manner and may invade the other parts of the body where they can form tumors known as metastases. Currently, metastases are the main cause of cancer deaths. (Dillekås et al., 2019; Hsu et al., 2019; Owyong et al., 2019; Wang et al., 2018) Though the primary cause of why some tumors metastasize and others do not, remains undetected. There is a group of cells, which directly bridges the primary tumor and metastatic tumor sites in the body. These cells are named circulating tumor cells (CTC). CTC are an infinite source of new information on tumor development in time. We believe more straightforward characterization of CTC on the molecular level could change the way how we think about the cancer in general.

The universal approach "one drug fits all" has been found to become ineffective in the term of toxicity risks and treatment failures for various diseases. Instead, individual patient approach in disease management has become more preferred. (Matsuzaki et al., 2020; Groisberg et al., 2018; Tsongalis, 2018; Cho et al., 2012) The point of view, how to plan patients' cure, has been developed to be more individual, known as personalized or precision medicine which has been referred as "the process of tailoring medical treatment to the individual characteristics of each patient; to classify individuals into subpopulations, that differ in their susceptibility to a particular disease of their response to a specific treatment, so that preventive or therapeutic interventions can then be concentrated on those, who will benefit, sparing expense and side effects for those who will not." along with "The molecular methods that make personalized medicine possible, include testing for variation in genes, gene expression, proteins, and metabolites as well as new treatments that target molecular mechanisms. Test results are correlated with clinical factors – such as disease stage, prediction of future disease status, drug response, and treatment prognosis – to help physicians individualize treatment for each patient." (Martínez-Jiménez et al., 2020; Maier, 2019; Shalowitz et al., 2019; Coleman and Tsongalis, 2017; Rinaldi, 2016; Silbermann, 2016)

Application of personalized medicine includes testing of newly developed molecules targets from whole molecular pathways through one mutated protein and aims to segment heterogeneous subset of patients whose response to therapeutic intervention within each subset is homogenous. (Crisci et al., 2019; Wang and Deisboeck, 2019; Dugger et al., 2018) Molecular-genetic methods and innovative technologies are useful tools to apply these personalized principles in clinical oncology, nowadays.

Tumor classification based on molecular characterization helps guide treatment of patients. The philosophy of personalized medicine has found to stop searching for one drug to treat all patients with a specific indication. It is necessary to focus on differencies in patients with the same disease who need to be treated differently, and to identify the best treatment for these patients and appropriate method to monitor the disease in a long-term perspective. (Martínez-Jiménez et al., 2020; Chen et al., 2019; Goldhammer et al., 2019; Kankeu Fonkoua and Yee, 2018)

Recently, the most frequently used tests include genetic alterations testing (e.g. TP53 gene) (Fortuno et al., 2020; Azzollini et al., 2018; Campo et al., 2018), mismatch repair genes testing (e.g. MLH1, MSH2, MSH6) (Antonarakis et al., 2019, Xavier et al., 2019; Zhao et al., 2019) overexpression and/or amplification of the genes (e.g. ESR, PGR, HER2) (Schaffar et al., 2019; Gupta et al., 2018; Kriegmair et al., 2018) or mutations predicting drug effectivity (e.g. KRAS, BRAF) (Bylsma et al., 2019; Midthun et al., 2019; Deshwar et al. 2018). The most updated information regarding new genes modifications may be found in The Cancer Genome Atlas (TCGA) of the National Cancer Institute (The Cancer Genome Atlas Program, 2019)

Regular testing of these genes' status could offer real-time monitoring of individual tumor development. The way how to reach this target could be connection of patients' liquid biopsy (LB) with molecular-genetic analysis. CTC and cell-free tumor deoxyribonucleic acid (ctDNA) are tumor markers obtained from blood of cancer patients. Both of them are bringing a new hope into the cancer field how to use "liquid" information in the process of therapy choice and therapy effect monitoring. Current research has signified combined analysis of CTC and ctDNA

increases sensitivity and provides real-time information about individual patients' disease status. (Gorges et al., 2019; McNamara et al., 2019; Ye et al., 2019; Onidani et al., 2019; Mohan et al., 2018)

Thus, CTC represent rare cells which were previously a part of tumor tissue, were shedded from it, survived in circulation and are still able to form metastases. Since CTC are present in patients' blood, they are a suitable tool for regular patients monitoring. After their enrichment several scenarios are available regarding their processing. We are able to observe their consecutive behaviour (growth, division, differentiation or ability to be part of/ to create clusters) or compare them with known tumor features from biopsy. This information can significantly contribute to overall picture of the patients' disease in real-time. (Amantini et al., 2019; Heeke et al., 2019; Chen et al., 2019; Pereira-Veiga et al., 2019; Jakabova et al., 2017)

Although wide spectrum of tumors has been studied for CTC, this thesis is focused preliminary on breast cancer (BC) patients. Population of CTC in blood system is very low. Their enrichment from blood is a crucial step which makes their characterisation even more challenging. In addition, we hypothesized that viable CTC can offer more information about cancer patients' disease status in comparison with the fixed ones. We were able to enrich viable CTC, cultivate them *in vitro* and monitor their behaviour and stage by cytomorphologic methodologies. Additional CTC- features could be obtained by gene expression analysis of tumor- and chemoresistance-associated genes. These analyses are performed regularly during treatment process. Results from these tests are then individually evaluated, correlated with current primary tumor characteristics and compared to patients' clinical outcomes. Long-term CTC monitoring enables to study both prognostic and predictive potential of CTC.

Breast carcinoma belongs to the most extensively studied areas within the wide spectrum of cancer diseases. Large international meta-analyses have been focused on CTC role in this cancer type. Recently, prognostic relevance of CTC in both adjuvant treatment and metastatic settings in breast carcinoma diagnosis has been determined. Outcome of these intensive researches confirms CTC position in the

field of biomarkers to be widely used in clinical practice. (Banys-Paluchowski et al., 2019)

The introduction is consists of chapters that provide insight to current knowledge about cancer and CTC. It explains cancer disease in general and then focus on process of carcinogenesis, the form of cancer cells. Another chapters are dedicated to cancer progression which includes actions needed for tumor growth, cancer cells release into circulation and development of metastasis which are essential mechanisms for CTC. The next chapter is focused on BC because most of the research was focused on this type and therefore more information about this disease need to be part of this dissertation work. This part includes risk factors, genes contributions, types and their treatment options. The important part in diagnosis and monitoring proces maintain biomarkers that can be also obtained from LB, since CTC are candidates for becoming biomarkers. The next chapter is subsequently dedicated to CTC, their enrichment, identification and value confirmed by research studies. The last chapter of introduction discusses resistence to anticancer drug because this phenomenon causes chemotherapy failure and continue of tumor growth.

1.1. Cancer diseases

The World Health Organisation (WHO) reported that cancer is the second leading cause of death, nearly 1 in 6 deaths is due to cancer. Lung, liver, colorectal (CRC), stomach and BC are the most common causes of death worldwide, respectively. (Cancer, 2018) As communicated newly, cancer became the leading cause of death in some high- and middle-income countries. Those countries include Sweden, Canada, Chile, Argentina, Poland and Turkey. (Dagenais et al 2019) WHO has also documented that 30-50% of all cancer events are preventable. Aside pediatric cancers, it usually takes decades until cancer develops. (Reiter et al, 2018) Risk factors contributing to tumor diseases development have already been revealed and need to be omitted for decreasing cancer occurrence. Tobacco use belongs to the most dangerous activity which is strongly connected to cause many types of cancer, including lung, oesophagus, larynx or mouth. Second-hand smoking has been proven to cause lung cancer and smokeless tobacco causes oral, oesophageal and

pancreatic cancer. Risk of cancer is also increased by alcohol consumption, often combined with tobacco. Alcohol-related tumor diseases include CRC, BC or liver cancer. Moreover, imbalanced physical inactivity in combination with dietary factor can support development of obesity-related cancers like oesophagus, pancreas, CRC, endometrial or kidney cancer. There are also non-modifiable factors such as age, race or family history that it is not possible to alter. In addition, some harmful environmental factors are also integral part of our lives, which can be rarely affected by individuals. Infections, radiation and environmental pollution are among the most important ones. (Gomes et al., 2020; Wang and Zhang, 2020; McKenzie et al., 2016; Midha et al,2016; Olver, 2016)

1.2. Cancer cells

Cancer disease is a result of a normal cell to a malignant cell transformation by a process called carcinogenesis. A lot of factors can contribute to this process. It includes genetic and metabolic transformation which is necessary for cancer initiation. Malignant character and behaviour of cancer cells (growth, proliferation, migration, and invasion) requires significant changes in metabolism to provide the sufficient energy supply.

1.2.1. Carcinogenesis

Carcinogenesis process has been intensively studied for decades. The process may be divided into three phases: Neoplastic transformation, Genetic / Metabolic Facilitative and Malignant. (Figure 1) These phases cover processes included in oncogenic transformation, metabolic transformation and malignancy.

The relationship between cell's activity and metabolism has been revealed as important part of carcinogenesis mechanism understanding. Costello and Franklin referred axioms of cell activity and cell metabolism for defining these principles.

1. The existing intermediary metabolism of a cell provides the bioenergetic/synthetic/catabolic requirements that are essential for the manifestation of the cell's current activity (e.g., function, growth, proliferation, differentiation).

2. When the activity of a cell changes, its metabolism must also be altered to provide new bioenergetic/synthetic/ catabolic requirements for the cell's changing activity.
3. Malignant cells are derived from normal cells that have undergone genetic transformation to neoplastic cells that have malignant potential.
4. Manifestation of the malignant potential of the neoplastic cell necessitates alterations in its metabolism (i.e., metabolic transformation) to provide the bioenergetic, synthetic, and catabolic requirements of malignancy.
5. In the absence of the metabolic transformation, the neoplastic cell will not progress to a malignant cell with its manifestation of malignancy. Conversely, the metabolic transformation, in the absence of the genetic transformation to a neoplastic cell, will not cause malignancy.
6. Genetic transformations and proteomic changes have little relevancy if the genetic/proteomic alterations are not manifested as changes in cell metabolism and function. The absence of identified genetic transformations and proteomic changes does not demonstrate the absence of changes in cell metabolism and function.

Cancer initiation, in the term of genetic/metabolic concept defines cancer as a genetic disease, requiring series of gene alterations, which produce mutations that activate oncogenes, mutations that inactivate tumor suppressor genes and influence genes stability (leading to increased mutation rates of other genes) known as oncogenic transformation. (Ebron et al., 2019; Böttcher et al., 2015; Volgenstein and Kinzler, 2004). Results of these events are neoplastic cells (genetically transformed cells which have malignant ability potential). This part of carcinogenesis is called a neoplastic transformation phase. (Sewastianik et al., 2019; Coleman, 2015)

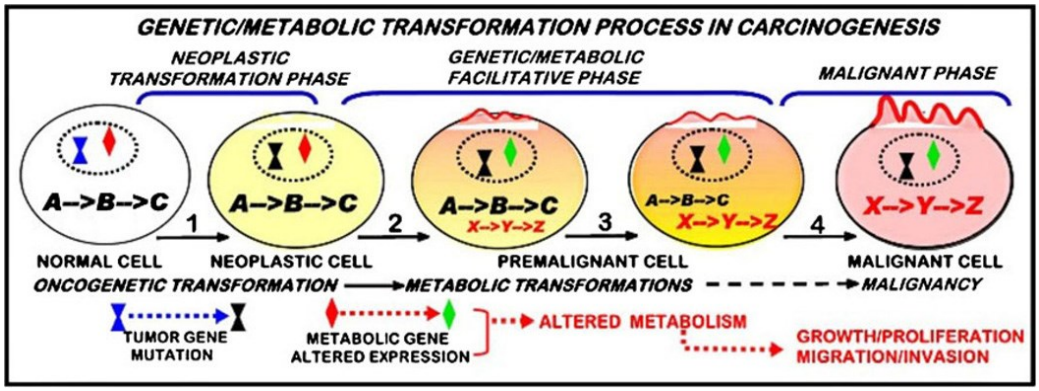

Figure 1.: Schematic representation of genetic/metabolic transformation proces of carcinogenesis. (adapted and modified from Costello and Franklin, 2012)

The next step to achieve fully malignant cells includes genetic and metabolic events occurred via oncogenic-initiated cascading and progressing signalling pathways and factors. Process is illustrated in Figure 1. (Costello and Franklin, 2012)

This multistep process includes also epigenetic changes which develop in cancer cells. The level of epigenetic changes increases with time and the changes are stable because despite toxicants' removal (arsenic exposure) after malignant transformation, both malignant phenotype and epigenetic changes remains. Epigenetic modification has also been connected to gene expression changes which can correspond to malignant phenotype progression. (Futscher et al., 2013; Chang et al., 2010)

1.3. Tumor growth (cancer progression)

Malignancy is presented by increasing mass of malignant cells which is following different oxygen gradient exposure from normoxia through hypoxia to anoxia. Under these limited conditions of lacking nutrients and limited removal of wasteful metabolic products are quiet and direct necessary activities. (Costello and Franklin, 2012; Hanahan and Weinberg, 2011; Alqawi et al., 2007; Dang and Samenza, 1999) In fact, tumor growth is essential for successful invasion and spread of disease through body. Both angiogenesis and lymphangiogenesis belong to the most important factors in this process. (Figure 2)

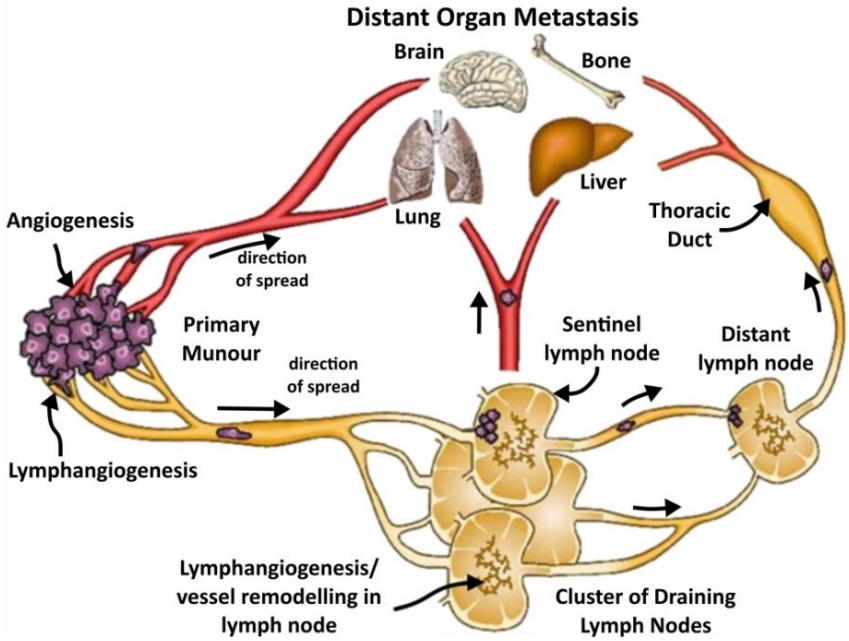

Figure 2.: Schematic representation of potential metastatic routes via lymphatic vasculature and lymph nodes (yellow) and blood vessels (red). (adapted from Achen and Stacker, 2008)

1.3.1. Angiogenesis

Growth of blood vessels, known as angiogenesis, belongs to one of the most important processes in our body. These capillaries are used for nutrients and metabolites exchange by diffusion mechanism. In general, angiogenesis can be affected by our life style habits. Therapeutic influences of this mechanism have been found to be effective in many diseases (cardiovascular diseases, wound healing, rheumatoid arthritis, etc.). (Ondrovics et al., 2017, Bartolucci et al., 2014)

Assumption that angiogenesis process is essential also for tumor growth and metastasis was proposed many years ago. Group of Folkman in collaboration with his colleagues (Sherwood et al., 1971) performed several experiments to demonstrate dependency of tumor invasive growth on neovascularization, including tumor angiogenesis in rabbit eye. They observed angiogenesis is used by tumors from 1-2 mm^3 of size for their growth and development. From the avascular environment of cornea, the slow growth of tumor at a linear rate was reported but after vascularization exponential development was observed. Moreover, concept of tumor dormancy caused by neovascularisation prevention was revealed. Viable and mitotically active tumor cells were in the aquaneous fluid of the rabbit eye up to 6 weeks. They were avascular and limited size. After their implantation into

environment full of blood vessels, they induced neovascularization and rapid growth. In addition, tumor cells can also survive in the form of dormant population which is characterized as a balance between proliferating cells near the surface of the spheroid and dying cells in the centre. (Ribatti, 2008) Pre-cancerous neoplasm, such as "carcinoma *in situ*" or "intraepithelial neoplasia" lesions can be surgically removed as benign tumors. These ones are localized, do not spread and necessary nutrients and oxygen are delivered from capillaries placed under the basement membrane of the epithelial layer via diffusion. If cell proliferation and apoptosis are relatively balanced the cells can be dormant for years. In the case of prostatic intraepithelial neoplasia (PIN) diagnosis, tumors may be dormant for years or progress. Activity of those lesions types is found to remain questionable. (Bielenberg and Zetter, 2015)

In tumor, angiogenesis is regulated by different types of regulators in the affected tissue. Hypoxia's environment has been considered as a critical factor in this process. Tumor is able to overcome oxidative stress and deficiency of oxygen-dependent energy production caused by this condition. Tumor and its miroenvironment is capable to stimulate new blood vessels formation which is attributed to vascular endothelial growth factor (VEGF). It has been found out hypoxia-inducible factor, (HIF)-1α, is the main compoment of molecular hypoxia-induced angiogenesis initiation. It has been observed HIF-1α is the critical element. While HIF-1α is continuously synthetized and degraded during normoxic condition, its stabilization, accumulation and dimerization with HIF-1β in the cytoplasm has been observed during hypotoxic conditions. This dimer is capable to control expression of many genes, including VEGF. Other important supporting components resulted from hypoxia conditions are cytokines (such as interleukin IL-8) or other growth factors (such as Platelet Derived Growth Factor - PDGF). Together with VEGF-A then contribute to angiogenesis by stimulation of Vascular Endothelial Growth Factor Receptor (VEGFR) on the endothelial cells surface. (Aguilar-Cazares et al., 2019; Choi and Park, 2017; Masoud and Li., 2015)

1.3.2 Lymphangiogenesis

Lymphatic system is essential part of our body responsible for tissue pressure regulation and as important component of the immune system. Lymphatic capillaries go through most of our tissues apart from e.g. cartilage, epidermis, nails, hair, retina or cornea which belong to avascular group of tissues. (Cueni and Detmar, 2008) Lymphatic vessels consist of lymphatic endothelial cells, opened into tissue periphery which attach to the extracellular matrix by filaments. (Gerli et al., 2000) Drained lymph fluid is passed through lymphatic capillaries into collecting vessels or it is released into the blood stream through lymph nodes cascade. (Mumprecht and Detmar, 2009) Lymphagiogenesis does not occur in adults just in exception of endometrium changes during pregnancy or pathological state such as repair processes, chronic inflammation or tumor growth. (Cueni and Detmar, 2008; Proulx et al., 2007; Red-Horse et al., 2006)

For successful lymphagiogenesis action, several growth factors are required. This group includes vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D) which bind to vascular endothelial growth factor receptor-3 (VEGFR-3) on lymphatic endothelial cells, hepatocyte growth factor which binds to c-met receptor or angiopoietin-1 which binds to Tie2 receptor (Mumprecht and Detmar, 2009; Morisada et al., 2005; Kajiya et al., 2005; Tammela et al., 2005) VEGF-C have become the most studied lymphagiogenesis factor in the manner of BC, melanoma, CRC, endometrial or ovarian cancers. Expression of VEGF-C in tumor or tumor-associated cells correlated with lymph node metastasis and poor prognosis. (Mumprecht and Detmar, 2009; Rinderknecht and Detmar, 2008; Pepper et al., 2003)

Lymphagiogenesis is a serious problem in cancer patients because it is associated with tumor invasion and metastasis. It occurs in tumor periphery and the inner-tumor cell mass. Tumors often metastasize to their regional lymph nodes via lymphatic vessels. Lymph node status is used to estimate survival and to choose appropriate clinical management strategy. (Mumprecht and Detmar, 2009; Van der Auwera et al., 2006; Dadras and Detmar, 2004)

1.3.3 Stem cells and their role in cancer

One of the current questions which have to be answered is where are the cancer cells coming from. Therefore, the role of stem cells dispered in the adult tissues still remains a matter of debate.

Stem cells are characterized by preserved ability to divide thanks to self-renewal. They are able to generate mature cells of appropriate tissue through differentiation process. Their incidence differs between tissues but as reported earlier they are rare. (Geraili et al., 2018; Reya et al., 2001) They need specific environment, factors and cytokines to survive. (Aponte and Caicedo, 2017; Guo et al., 2016; Wu and Izpisua Belmonte, 2016; Ouspenskaia et al., 2016) Stem cells have an important mission in keeping homeostasis within organism. They are responsible for replacing cells and maintaining functions of organ by self-renewing and regulating their quantities in environment of physiological and abnormal conditions. (Aponte and Caicedo, 2017; Shenghui et al., 2009; Alison et al., 2002) Adult stem cells present in tissues have partially limited differentiation potential. (Lane et al., 2014; Scadden et al., 2014; Morrison and Spradling, 2008) Despite different locations, all stem cells share similar characteristics such as long cell cycling, self-renewal capacity, microenvironmental protection, genome repair abilities or undifferentiated status combined with differentiating potential. (Aponte and Caicedo, 2017; Alison et al., 2002) These cells are one of the main members of hierarchical organization known as Stem Cell Systems (SCSs) which are the part of almost all major organs in different quantities. (Aponte and Caicedo, 2017; Gage and Temple, 2013)

Following sections are present in SCSs: the basal, the transmit-amplifying and the differentiation. (Alison et al., 2002) Basal compartment of this system is a residence of stem cells. This environment, together with other factors (extracellular matrix, oxygen, etc.) contributes to the stem cells survival and maintenance. (Lane et al., 2014) Stem cells progenies then fill transit-amplifying compartment. Cells which are part of this section are in transient state. They have shorter cells cycle and divide rapidly. The differentiation compartment is residence of differentiated cells that are ready to perform normal tissue or organ function. Under specialized conditions, these cells can also dedifferentiate and add into stem cells pool. SCSs thus have

become highly regulated parts where balance between self-renewal and differentiation activity is maintained. (Aponte and Caicedo, 2017)

Genes responsible for self-renewal have been found to work as oncogenes in comparison with differentiating ones. Tumors consist of heterogeneous cells. Normal stem cells and cancer / tumorigenic stem cells are able to create phenotypically different cells. Cancer cells heterogeneity is also partly caused by tumor environment. Tumors mass may be increasing also due through mutations that upset normal pathways and then culminate in circulating stem cells (CSCs) formation. (Clarke and Fuller, 2006; Reya et al., 2001). According to evidence the progenitor cells pools in the tumors are able to revert back to CSCs state by various ways, such as epithelial-mesenchymal transition. (Aponte and Caicedo, 2017)

1.3.4 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a process of biochemical modification to achieve mesenchymal phenotype of epithelial cell. Character of mesenchymal phenotype cells is presented typically by enhanced migration, invasiveness, increased resistance to apoptosis and elevated secretion of extracellular matrix (ECM) components. (Lee and Nelson, 2012; Kalluri and Neilson, 2003) Final step of EMT consists of underlying basement membrane degradation so that the mesenchymal cells can migrate from the epithelial layer of its origin. (Angadi and Kale, 2015; Kalluri and Weinberg, 2009; Lee et al., 2006)

The term “epithelial-mesenchymal transformation” meaning a conversion from epithelial to mesenchymal state was used by Elisabeth Hay as first. She used a chick primitive model to determined dramatic phenotypic changes performed. (Kalluri and Weinberg, 2009) Because of the reversibility process and prevention to neoplastic transformation confusion, “transformation” was renamed to “transition”. A reverse process of mesenchymal-epithelial transition (MET) aims to achieve epithelial phenotype of cells. (Angadi and Kale, 2015; Lee and Nelson, 2012; Kalluri and Weinberg, 2009) Epithelial cells have apicobasal polarity, they held together by intracellular adhesion complexes on the basement membrane. They are not able to migrate and express epithelial markers. On the contrary, mesenchymal

phenotype is associated with absence of stable cell junctions, anterior/posterior polarity, increased matrix degradation and expression of mesenchymal markers. (Vergara et al., 2016; Kalluri and Weinberg, 2009) EMT process initiation requires the loss of cell-cell adhesion, alteration in expression of cell-surface protein, production of ECM degrading enzymes, transcription factors activation or expression and reorganisation of cytoskeletal proteins. (Thiery, 2003; Thiery, 2002) Reorganisation of cytoskeleton, loss of epithelial cell proteins such as E-cadherin, claudins or occluding and elevation of mesenchymal markers such as N-cadherin, vimentin or fibronectin belong to main characteristics of EMT process. (Trimboli et al, 2008; Huber et al., 2005) (Figure 3)

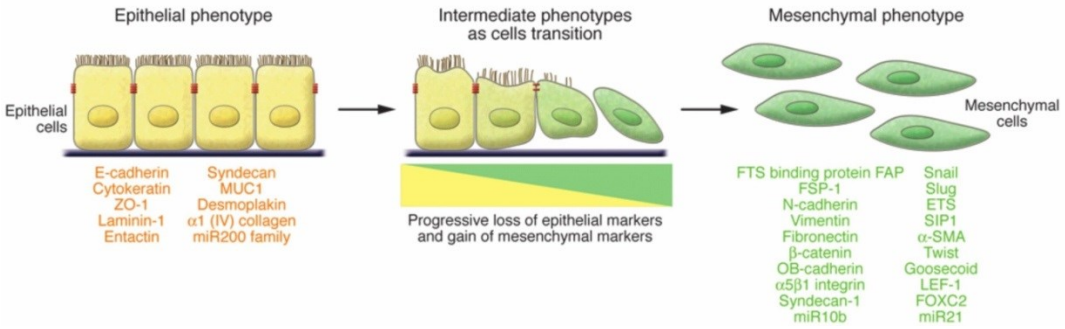

Figure 3: Schematic representation of functional transition from epithelial cells through intermediate phenotypes into mesenchymal cells. Characteristic epithelial and mesenchymal markers are shown below the corresponding phenotypes. Intermediate phenotype of EMT defines cells with both dinstict markers expression. ZO-1, zona occludens 1; MUC1, mucin 1, cell surface associated; miR200, microRNA 200; SIP1, survival of motor neuron protein interacting protein 1; FOXC2, forkhead box C2. (adapted from Kalluri and Weinberg, 2009)

EMT process is divided into three different subtypes according to biological context in which they occur (Figure 4). The first type, known as type 1 EMT, is performed during embryo implantation and initiation of placenta formation. Primitive streak is generated in the epiblast layer. Epithelial cells in this tissue express E-cadherin and exhibit apical-basal polarity. The epithelial-like cells undergo programmed changes governed by specific expression of proteins linked with cell migration and differentiation. This form of EMT is presented during embryo implantation, placental development, gastrulation and organ development. (Lee and Nelson, 2012; Kalluri and Weinberg, 2009)

The second form of EMT, type 2 EMT, is associated with wound healing and organ fibrosis. Role of this mechanism is repairing tissue after trauma and inflammatory

damage by generating fibroblast and other cells necessary for reconstruction. This type of EMT can also lead to organ damage in the case of primary insult persistence. (Lee and Nelson, 2012; Kalluri and Weinberg, 2009)

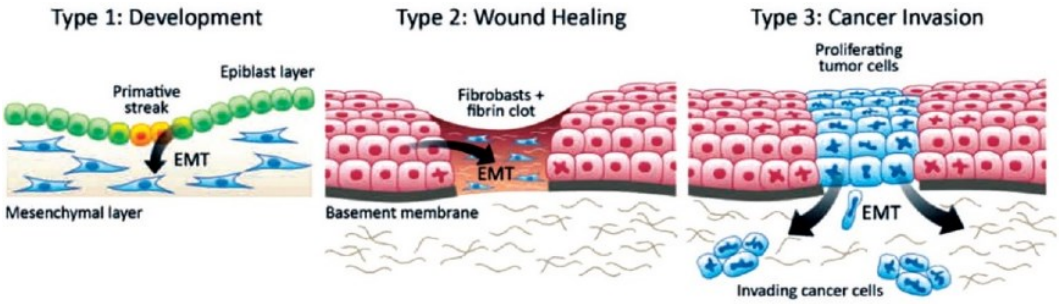

Figure 4.: Schematic representation of three types of EMT – development, wound healing and cancer invasion. (adapted from Angadi and Kale, 2015)

The last EMT subtype is known as Type 3 EMT. This one is associated with cancer progression and metastasis. Researchers have proposed EMT activation is critical mechanism for malignant phenotype acquisition. This process is believed to be responsible for cancer dissemination. Observation of EMT activation in human tumors is often related to disease progression and drug resistance. It also causes morphological and genetic changes result from elevated migratory and invasive capabilities. The cancer cells are not equal in tumor mass. Epithelial and mesenchymal subtypes can coexist and cooperate with each other. These changes alter the tumor suppressor genes and oncogenes which regulate EMT system. It is followed by basement membrane invasion, metastasis to other locations introducing the terminal step of carcinogenesis process. Cancer cells derived during EMT process are then establishing secondary colonies which are histopathologically similar to original tumor. These cells have not longer mesenchymal phenotype which is the result of MET mechanism. (Vergara et al, 2016; Angadi and Kale, 2015; Kalluri and Weinberg, 2009)

Regulation of transcription factors activity and expression that induce EMT are dependent on signalling pathways. Signal is transmitted though intracellular kinase cascades to induce transcription factors that include e.g. transforming growth factor β (TGF-β), bone morphogenic protein (BMP), fibroblast growth factor (FGF), PDGF, epidermal growth factor (EGF), Wnt, Notch, Sonic Hedgehog (Shh) and integrin signalling. Signals tend to be cell- and tissue type-specific. Different state,

type or environment of cells may result from different reactions with diverse sensitivities. (Gonzalez and Medici, 2014; McCormack and O'Dea, 2013; Espinoza and Miele, 2013; Heldin et al., 2012; Thiery et al., 2009) Signaling pathways which are involved in EMT are showed in Figure 5.

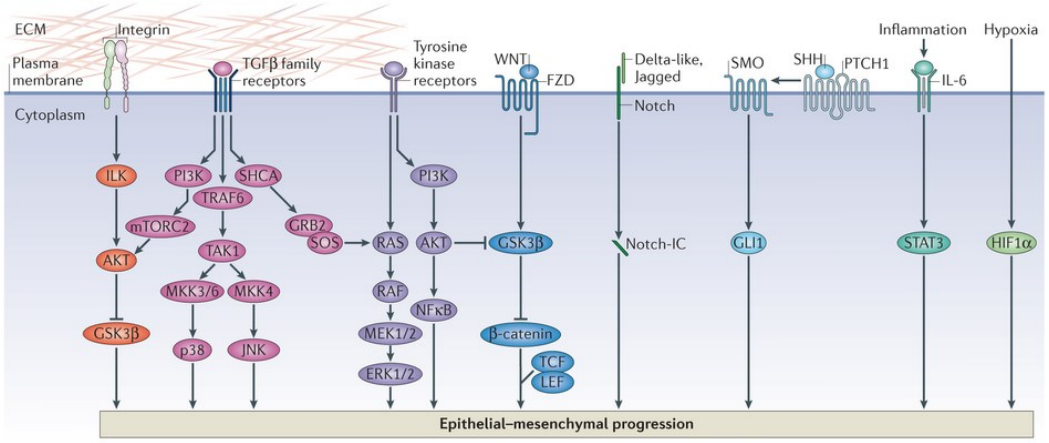

Figure 5.: Schematic representation of signalling pathways involved in EMT. ECM, extracellular matrix; TGFβ, transforming growth factor-β; FZD, frizzled; SMO, smoothened; SHH, sonic hedgehog; PTCH1, patched 1; IL-6, interleukin-6; ILK, integrin-linked kinase; GSK3β, glycogen synthase kinase-3β; mTORC2, mammalian TOR complex 2; TRAF6, TNF receptor-associated factor 6; TAK1, TGFβ-activated kinase 1; MKK, MAPK kinase; JNK, JUN N-terminal kinase; SHCA, SRC homology 2 domain-containing-transforming A; GRB2, growth factor receptor-bound protein 2; SOS, son of sevenless; NFκB, nuclear factor-κB; TCF, T cell factor; LEF, lymphoid enhancer-binding factor 1; GLI1, HH signalling, glioma 1; STAT3, Janus kinase (JAK)–signal transducer and activator of transcription 3; HIF1α, hypoxia-inducible factor 1α. (adapted from Lamouille et al., 2014)

1.3.4.1 Epithelial-mesenchymal transition markers with clinical relevance

It turns out, that markers associated with EMT may have impact on cancer patients. Lower level of E-cadherin expression is one of the typical signs of the ongoing EMT transition. Secretion of β-catenin into the cytosol and nucleus, possibly, is often connected with reduced E-cadherin expression. This association was found in triple-negative and basal-like types of BC samples by immunohistochemical analysis. (Vergata et al., 2016; Geyer et al., 2011; Jeanes et al., 2008) On the other hand, high expression of E-cadherin can be a positive indicator for disease-free and overall survival in squamous cell lung carcinoma patients but abnormal expression of this marker is also associated with poorer differentiation degree in the case of esophageal cancer. (Xu et al., 2014; Zhang et al., 2013)

Decreased level of E-cadherin is often associated with elevated N-cadherin expression. Its overexpression has been associated with poor differentiation, reduced overall survival and advanced Tumor, Nodes, Metastasis (TNM) stage in numerous cancer types. (Hiu et al., 2013; Di Domenico et al., 2011; Lascombie et al., 2006) Snail, Slug and Twist proteins have been found to be correlated with poor survival, worse outcome and advanced stages in more types of cancer. In the case of BC, Snail, Twist and Twist/Snail ratio have been revealed as predictive for metastatic and distant relapses. (Vergata et al., 2016; Muenst et al., 2013; Tran et al., 2011; Jouppila-Mättő, 2011)

Expression of vimentin (VIM) is related to EMT, too. Aggressive behaviour, metastatic potential, chemoresistance, high histological grade and poor prognosis can be predicted by determination of its expression alone or in combination with other markers in several cancer types. In BC, higher expression of VIM was detected in triple-negative BC in comparison with other BC types and correlated with high Ki67 expression, nuclear grade, poor prognosis and younger age. (Vergata et al., 2016; Karihtala et al., 2013; Satelli and Shulin, 2011)

1.4 Metastatic models

In the majority of the cancer cases, tumor-associated deaths are caused by metastasis. Overall, metastatic process is not very effective; it is driven by sequence of mechanical and molecular obstacles. Most of the cancer cells are not able to create tumors at the distant sites. Although period to new distant tumors establishment is individual, certain cancer types exhibit organ specific metastatic potential. (Figure 6) Solid tumors are likely to metastasize to preferred sites. BC often metastasizes to lung, liver, bone and brain; CRC to liver and lung; or lung adenocarcinoma likely metastasize to bone, liver and brain. It has turned out that the most frequent metastatic sites are found in lungs, liver, bones or brain. (Chen and Ahmad, 2019; Li et al., 2019; Qiu et al., 2018; Azevedo et al., 2015; Erez and Coussens, 2012) Mostly, initial delivery and arrest of tumor cells to specific organs is expected to be mainly based on blood flow circulation and/or lymphatic drainage. Some of the belligerent cancers spread high numbers of tumor cells to the circulation every day. Both streamlines of blood circulation, arterial and venous,

can contribute to tumor cells delivery differently. Although arteries are usually flexible which allows them to regulate the blood flow indirectly, veins are not shaped by high pressure and act as a blood reservoir. Tumor cells, located in circulation, are guided by blood circulation while they pass through specific organs. (Azevedo et al., 2015)

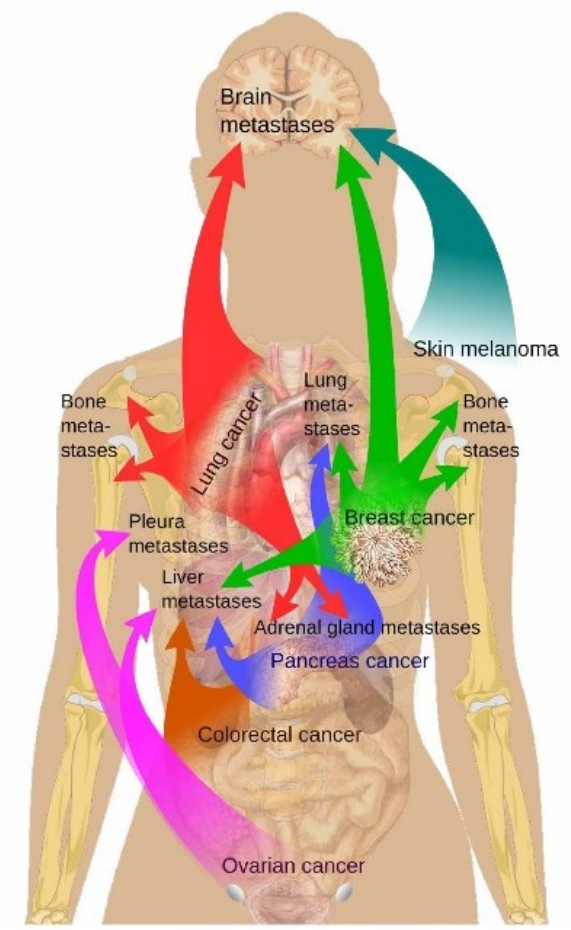

Figure 6: Common sites of metastasis for different primary sites and cancer types. (adapted from Liver Metastases, 2019)

Metastatic model known as “seed and soil” hypothesis was suggested by Stephen Paget based on BC patients with specific metastasis. Cancer cells, as seeds, colonize specific distant organs, as soil, where they are able to survive and proliferate. (Paget, 1989) The development process of these metastatic “seeds” into larger volumes is controlled by specific conditions of organ and its microenvironment. The majority of CTC will finally die in distant organs or before landing at distant organs. (Paterlini-Bréchot, 2014; Nguyen et al., 2009) Mechanical factors have been revealed to be a key determinant of cancer cells settlement and survival.

(Chambers et al., 2002) Five leading metastatic sites (lung, bone, lymph node, liver and pleura) are the most seeded organs for disseminating tumor cells during colonisation because of the “mechanical” circulation patterns. (Azevedo et al., 2015)

A relatively new model of metastasis considers blood flow in combination with organs filtering effect. In general, the model stresses the role of organs with high capillary vasculature that purify and filter blood. (Scott et al., 2014; Scott et al., 2012) It is hypothesized that initial delivery and arrest of tumor cells is primarily regulated by physical parameters of cells. Cancer cells circulation trapping because of their size is proved by practical experiments. (Azevedo et al., 2015; Steinbauer et al., 2003; Mook et al., 2003) E.g. live imaging of brain revealed “big” tumor cells (mean diameter: 12-20mm) trapped by capillaries (diameter: 3-8mm). (Kienast et al., 2010) These mechanisms also depend on location and blood vessels diameter in particular areas. Although size-based restriction is an important event, active adhesion between metastatic cells and vascular wall is required for arrest and metastasis establishment. Most probably, physical arrest in combination with active adhesion result from metastatic cells stabilization. (Azevedo et al., 2015; Glinskii et al., 2005; Enns et al., 2004)

Survival and metamorphosis of tumor cells after entering to circulation depends on their physicochemical interactions with other parameters that contribute to their successful arrest and metastasis establishment. Three of them, blood constituents, vascular walls and broader vascular flow pattern, play essential role in this mission. Tumor cells have developed various strategies to overcome problems with their size which is larger in comparison with other cells. Shear stress and flow velocity have found to be toxic for cells. Cancer cells located in the circulation preferentially arrest in the small blood vessels with curved, branched and stretched microvasculature that are usually found in lungs, liver, brain or bone (Guo et al., 2014; Disibio et al., 2008) They may also use their deformability to move through capillaries and clog them. Coagulation with platelets has found to become helpful because they are able to surround tumor cells, protect them from immune system and alter shear stress. Result of agglomerate formation is its rolling on the endothelial surface which favours interaction between carcinoma and endothelial

cells through specific adhesion types. (Azevedo et al., 2015; Amini et al., 2014; Burdick et al., 2004)

1.5 Breast cancer

BC diagnosis belongs to the most frequent oncologic diseases and the most common cancer in women worldwide. BC risk has an increasing trend, but survival rates improved, in general. Main reasons include earlier and localised diagnostics, medical care access and progressive treatment strategies improvement. (Siegel et al., 2016)

1.5.1 Risk factors for breast cancer

Although high numbers of risk factors have been found to be partly responsible for BC manifestation in patients, their exact contribution is individual. They may be divided into those which cannot be modified (age, gender, family history) and those, that can (diet, smoking, physical activity). (Table 1) According to case-control studies provided in the Czech Republic, eight risk factors for manifestation of BC modification were revealed: the number of first-order relatives with breast carcinomas, the number of first- and second-relatives with any malignant disease, menarche age, the first birth age, number of breast biopsies, atypical ductal hyperplasia presence in biopsy sample, body mass index, and breast inflammation. (Novotný et al., 2016; Yang et al., 2011; McPherson et al., 2000

Table 1: Modifiable and non-modifiable risk factors of BC manifestation in patients. (adapted from Breast density: over 700,000 UK women living with 'hidden' breast cancer risk, 2014)

Non-modifiable risk factors	Modifiable risk factors
Age	Body weight
Gender	Physical activity
Personal cancer history	Alcohol use
Family cancer history and genetics	Smoking
Early menstruation and late menopause	Exposure to hormones (Pills, *in vitro* fertilisation, hormone replacement therapy)
Breast density	Pregnancy and breastfeeding
Breast condition	Radiation exposure

Standard determinants of BC contribution try to help women who may benefit from more intensive cooperation with clinicians to decide their personal risk. Not all of the women with risk factors have to develop tumor. Indeed, ordinary woman has about 10-15% chance of BC development but with cancerous family history the probability may be over 50%. (Chen et al., 2017)

Increasing age belongs between the most important non-modifiable risk factors in most cancer types, including breast. Incidence is usually rising until menopause. Then the increase is slowlier and it starts to decline with age. BC affects 100 times more women in comparison with men. Surprisingly, studies have supposed that tall stature can be associated with increased risk. Women taller than 175 cm have bigger risk to develop BC in comparison with those who are shorter than 160 cm. (Giovannucci, 2019; Chen et al., 2017; McPherson et al., 2000)

Relation between reproductive and hormonal factors and BC was firstly suggested by Bernadino Ramazzini more than 300 years ago when he noticed higher frequency of this disease in nuns compared with other women. (Horn and Vatten, 2017; Chen et al., 2017) According to new studies, both the first menstruation age and time when menopause starts influence BC risk. Similarly, pregnancy and breastfeeding can be also partially involved in developing BC. It has been found that women who give birth multiple times and have full-term pregnancy in younger age have lower risk of this disease in comparison with women who have never given birth and were pregnant in older age, respectively. Protective effects have also been found in breastfeeding. (Chen et al., 2017; Yang et al., 2011; McPherson et al., 2000)

Other modifiable factors are usually associated with peoples' lifestyle. Both physical inactivity and obesity result in unbalanced of energy between consumption and expenditure. Excessive amount of adipose tissue increases the risk of BC development and progression that is accompanied by overall and BC mortality. (Chan et al., 2014; Demark-Wahnefried et al., 2012; Friedenreich et al., 2010) Physical activity is usually connected with improved survival. (Hardefeldt et al., 2018; Ballard-Barbash et al., 2012; Ellsworth et al., 2012) Mortality risk of young women patients with BC was reduced in young inactive women. Specialised

mechanisms have been suggested for beneficial effects of physical activity in this disease. (Brenner et al., 2016; McPherson et al., 2000)

Dietary factors are strong candidates to influence BC process but evidence has not been clear, yet. Connection between diet and BC process has been the main subject of research, worldwide. Although the mortality risk is supposed to decrease with post-diagnostic fruit, vegetable, whole grain or protein consumption, it is to be increased with animal fat. Higher dietary fat consumption seems to influence BC risk, progression and recurrence through promotion of oxidative stress, hormonal dysregulation or inflammatory signalling. (Brenner et al., 2016; Makarem et al., 2013) Recent studies suggest dietary interventions such as caloric restriction or fasting-mimicking diet may be used not only as a prevention strategy but also help during BC treatment. (Caffa et al., 2020; de Groot et al., 2020; Laudisio et al., 2020; Shin et al., 2020)

1.5.2 Clinicopathological and molecular features of breast cancer

BC is usually divided into several subgroups based on clinicopathological parameters that include nodal status, tumor grade, tumor size or menopausal status in combination with markers determinating prognosis, prediction and treatment selection. (Castro et al., 2019; Vallejos et al., 2010; Cheang et al., 2009; Sotiriou et al., 2003)

1.5.2.1 Tumor staging and grading

BC presence is usually revealed through breast lump examination or mammography screening. Results of atypical mammogram contain micro-calcification; breast masses or architectural distortions and suspicious masses are usually painless and settled in neighbouring tissue. These discoveries are then undergoing biopsies or ultrasound survey to determine of pathological diagnosis and more accurate localization. Additional information about breast tumor can be achieved by additional medical tests such as breast and/or chest imaging, blood count, analysis of liver function, magnetic resonance imaging or computer tomography scan. (Kuppusamy et al., 2019; Telloni et al., 2017)

TNM classification system is used as common nomenclature of disease. It categorizes BC into several subgroups by analysis of primary tumor (T), regional lymph nodes (N), and distant metastasis (M). The TNM results stage groups which provide more on prediction of patients' outcome reflecting patients' tumors heterogeneity. Additional tumor characteristics are needed to complete more specific information but TNM classification remains the basis of BC diagnostic (Table 2). (Oluogun et al., 2019; Giuliano et al., 2017)

Pathological examination of tumors usually includes determining the rate of its differentiation. Similarities between normal breast cells and those of tumor are microscopically examined. Pathologists are focused on 3 factors – tubular formation, nuclear pleomorphism and mitotic activity, all of them are reflected in final grade decision. According to the signs, score from 1 to 3 (1-closer to normal; 3-different from normal) are assigned to each group. These values are then summed up and final number defines the grade. Tumors with final score 3-5 belong to grade 1 (well differentiated); score 6-7 are in grade 2; and score 8-9 means grade 3 (poorly differentiated). (Guiliano et al., 2017; Komaki et al., 2006) Lymph node status, tumor grade and tumor size parameters can be considered using the Nottingham prognostic index for invasive BC or Van Nuys prognostic index for ductal BC patients to survival or relapse risk estimation. (Novotný et al., 2016)

Table 2: TNM classification and stage in BC patients. (adapted from Giuliano et al., 2017)

Stage	**TNM**	**Stage**	**TNM**
0	Tis; N0; M0	**IIIA**	T1; N2; M0 T2; N2; M0 T3; N1; M0 T3; N2; M0
IA	T1; N0; M0	**IIIB**	T4; N0; M0 T4; N1; M0 T4; N2; M0
IB	T0; N1mi; M0 T1; N1mi; M0	**IIIC**	Any T; N3; M0
IIA	T0; N1; M0 T1; N1; M0 T2; N0; M0	**IV**	Any T; Any N; M1

IIB	T2; N1; M0 T3; N0; M0		

TX - primary tumor cannot be assessed

T0 – no evidence of primary tumor

Tis (DCIS) - Ductal carcinoma in situ (DCIS)

Tis (Paget) - Paget disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS) in the underlying breastparenchyma. Carcinomas in the breast parenchyma associated with Paget disease are categorized based on the size and charac-teristics of the parenchymal disease, although the presence of Paget disease should still be noted

T1 - Tumor ≤ 20 mm in greatest dimension

T2 - Tumor > 20 mm but ≤ 50 mm in greatest dimension

T3 - Tumor > 50 mm in greatest dimension

T4 - Tumor of any size with direct extension to the chest wall and/or to the skin; invasion of the dermis alone does not qualify as T4

pN0 - No regional lymph node metastasis identified or ITCs only

pN1 - Micrometastases; or metastases in 1-3 axillary lymph nodes; and/or clinically negative internal mammary lymph nodes with micrometastases or macrometastases by sentinel lymph node biopsy

pN1mi – Micrometastases (up to 200 cells from 0.2mm to 2mm)

pN2 - Metastases in 4-9 axillary lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in the absence of axillary lymph node metastases

pN3 – metastases in 10 or more axilliary lymph nodes; or in infraclavicular lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in the presence of one or more positive level I and II axilliary lymph nodes; or in more than 3 axilliary lymph nodes and micro/macro metastases by sentinel lymph node biopsy in clinically negative ipsilateral internel mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes

M0 - No clinical or radiographic evidence of distant metastases

M1 - Histologically proven metastases larger than 0.2 mm (pM)

*for more detailed criteria see Giuliano et al., 2017: “Breast Cancer—Major changes in the American Joint Committee on Cancer eighth edition cancer staging manual”; Lymph nodes are defined as pathological (pN), definition of clinical is not viewed here. ITC – isolated tumor cell

1.5.2.2 Molecular markers of breast cancer

The basic BC molecular subtypes are standardly evaluated by immunohistochemical diagnosis of hormonal and growth receptors. Estrogen receptor (ESR), progesterone receptor (PGR) and human epidermal growth factor receptor 2 (HER2) are used for primary tumor subtypes classification. In the cases of more precise characterization, other markers (e.g. Ki67, mammaglobin (MGB),

VEGFR, p53, Phosphatase and Tensin Homolog (PTEN)) are also used. (Baker et al., 2019; Firouzabadi et al., 2019; Guestini et al., 2019; Dai et al., 2016; Reigosa et al., 2016; Fulford et al., 2006)

ESR has become one of the most accepted biomarkers for classification of BC. This marker is usually present in 75% of BC tumors, which are likely well-differentiated, less aggressive and connected with better outcome after surgery in comparison with ESR negative ones. (Dunnwald et al., 2007; Putti et al., 2005) Predictive value of ESR is important in relation to the hormonal therapy receiving. Patients with ESR negative tumors rarely benefit from endocrine therapy but approximately 50% of patients with ESR positive tumors respond to anti-estrogen or aromatase inhibitors therapy. (Dowsett et al., 2006; Esserman et al., 2005) Together with ESR, also PGR status has been widely used for routine diagnosis of BC tumors. PGR positive tumors are positive in 65% - 75% of BC. PGR have found to be a helpful marker usually due to its relation to ESR. Although PGR positive tumors are rarely ESR negative, approximately 40% of ESR positive tumors are PGR negative. (Dai et al., 2016; Regan et al., 2006)

ESR and PGR are used for dividing tumors into four subgroups according to their expression: ESR+PGR+; ESR-PGR+; ESR+PGR- and ESR-PGR- (plus (+) represents positive; minus (-) represents negative). (Rakha et al., 2007) Defining of these categories belongs to first steps after diagnosis determination because of prognostic and predictive forecast. Both markers positivity occurs in 55%-65% BC tumors and their successful responsiveness to endocrine treatment has been observed in 75%-85% of these cases. ESR+PGR+ BC subtypes are usually associated with lower mortality rate, older age, smaller tumor size and lower grade. (Dunnwald et al., 2007; Rakha et al., 2007; Anderson et al, 2001) In contrast, ESR-PGR- tumor types represent 18%-25% of BC tumors and are often associated with heterogeneity, higher grade, lower overall survival, higher recurrence rate and though do not respond to endocrine treatment, but anthacycline and/or taxane-based preoperative therapy has usually shows a good response. (Bardou et al., 2003 Anderson et al, 2001; Ravdin et al., 1992) In addition, single positivity of one of the receptors, either ESR or PGR, comprises up to 17% of BC cases. They are more likely to be aneuploidic, of larger size, higher histological grade, and usually show

increased expression of genes associated with proliferation in comparison to ESR+PGR+ tumors. Their response to endocrine treatment is worse than in ESR+PGR+ BC tumors. (Rakha et al., 2007; Dunnwald et al., 2007; Arpino et al., 2005; Anderson et al, 2001)

Beside hormone receptors, a HER2 examination is routinely provided to classify BC subtypes. According to studies, HER2 positivity is usually associated with poor prognosis and better response to anthacycline-related regimens, taxane-combined regimens in comparison to HER2 negative tumors. (Gianni et al., 2009; Tubbs et al., 2009; Pritchard et al., 2008; Hayes et al., 2007) HER2 marker represents a target for new cancer drugs, in parallel. It has been also reported that tumors with HER2 positivity likely benefit from paclitaxel addition after adjuvant therapy (doxorubicin + cyclophosphamid) in node-positive BC irrespective of ESR status but ESR+HER2+ tumors have usually little profit from this treatment. (Dai et al., 2016; Hayes et al., 2007)

Based on immunochemical results, 4 main subgroups are classified using ESR and/or PGR in combination with HER2. Patients with hormone receptors negative tumors have generally worse prognosis than ESR and/or PGR positive types. Current studies report that ESR+/PGR+ HER2+ tumors have the best prognosis and also the best response to hormone treatment but ESR-PGR-HER2+ and ESR-PGR-HER2- tumor types often show aggressive behaviour, poor differentiation and poor outcome, as next response to hormonal treatment is rare. Other markers are newly used for getting more information on BC tumors. (Dai et al., 2016)

Beside HER2 also androgen receptor (AR) has been used in clinical diagnostic. This receptor is expressed in 90% of ESR+ and 55% of ESR- tumors. (Hu et al., 2011; Ogawa et al., 2008) Analysis of ESR-PGR-AR- (receptors negative) and ESR-PGR-AR+ (molecular apocrine) groups revealed that molecular apocrine tumors cover 13.2% of all BC cases and are usually marked by Ki67 positivity. These tumors have relatively good outcome, often comparable with ESR+PGR+ tumor type, generally in therapy including taxanes (Dai et al., 2016; Lakis et al., 2014; Farmer et al., 2005).

Ki67 is the most widely used marker of proliferation that is used mainly for neoadjuvant response or adjuvant therapy outcome prediction in BC. It is evaluated in combination with ESR, PGR and HER2. Classification of tumors into ESR+/PGR+HER2-Ki67+ and ESR+/PGR+HER2-Ki67- has revealed that subgroup with Ki67 positivity has been connected with poor outcome regardless of systemic therapy. (Dai et al., 2016; Miglietta et al., 2009; Cheang et al., 2009; Jacquemier et al., 2009; Viale et al., 2008)

1.5.3 Molecular subtypes of breast cancer

Various methods have been used to determine BC subtypes. In general, four main subgroups were defined through extensive studying of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), microRNA and protein levels: Luminal A, Luminal B, HER2-enriched, and Basal-like types (Table 3). (Koboldt et al., 2012) Each tumor subgroup exhibits individual features, such as prognosis, incidence, therapy response, preferential metastatic organs or disease-free survival outcomes (Figure 7). (Cho, 2016; Parker et al., 2009) It has been also found out coexistence of more that one tumor subtype can be detected in the same patient. (Verma et al., 2016)

Table 3: Molecular subtypes of BC tumors. (according to Novotny et al., 2016; Cho, 2016)

Subtype	**Characteristics**				**Therapy**
	ESR	PGR	HER2	Ki67	
Luminal A (grade < 3)	+	+	-	Low	Endocrine
Luminal B (HER2-)	+	- or low	-	High	Endocrine ± CHT
Luminal B (HER2+)	+	Any	+	Any	CHT + anti-HER2+ endo
HER2 (non-luminal)	-	-	+	NA	CHT + anti-HER2
Basal-like (ductal)	-	-	-	NA	CHT

NA, not applicable; CHT, chemotherapy; endo, endocrine therapy; plus (+) represents positive; minus (-) represents negative

1.5.3.1 Luminal subtype

Luminal tumors are the most common subtypes of all BC cases. Luminal A and Luminal B types make up 90-95% of ESR and/or PGR positive and HER2 negative

tumors. (Prat et al., 2015) It has been found that Luminal A types usually show higher expression of ESR-related genes, lower expression of proliferation-related genes and are tent to be of lower grade in comparison with luminal B tumor types. (Sørlie et al., 2003) Prognostic analysis revealed relatively good prognosis in the case of luminal subtypes but Luminal B tumors have significantly worse prognosis and reccurence-free survival regardless of adjuvant systemic therapy than Luminal A even though Luminal B tumors show higher pathological complete response (pCR) rate following neoadjuvant therapy. (Cho, 2016; Ng et al., 2015; Prat et al., 2015; Sørlie et al., 2003) Various methods have been used to determine BC subtypes. In general, four main subgroups were defined through extensive studying of Anti-angiogenic approach works effectively for this tumor types based on progression-free survival improvement in metastatic BC (mBC) patients after receiving bevacizumab (anti-VEGF antibody) therapy in combination with paclitaxel. Although luminal tumors often poorly response to standard therapy, they response well to hormone medicine. Treatment response thus differs also between luminal tumors. (Brenton et al., 2005; Paik et al., 2004) Luminal A types may be treated with hormonal therapy only, a combined treatment including chemotherapy and endocrine therapeutics could be more effective in BC patients with luminal B subtypes. (Xiaofeng et al., 2015)

1.5.3.2 Human epidermal growth factor receptor 2-enriched subtype

Tumors with HER2 over-expression profile have been found in 15-25% of invasive BC cases. 40-80% of these subtypes harbour TP53 mutation and are more likely to be of grade 3. Hormone receptors (HR) can also play a significant role in HER2+ cancer cells behaviour. Among HER2-enriched subtypes, both increased disease-free and overall survival have been rather associated with HR+ tumors in comparison with HR- subtypes. (Cho, 2016; Xiaofeng et al., 2015; Arteaga et al., 2011; Perez et al., 2011) Similarly, for HR- /HER2+ group of patients significantly more cancer recurrences were observed. In HR-/HER2+ women, more first relapses in the brain and less in the bone occurred. (Iwamoto et al., 2012; Vaz-Luis et al., 2012) Although tumors with HER2 over-expression display poor prognosis, their pCR is higher than in luminal tumors after anthracycline and taxanes-based

neoadjuvant therapy regimens. (Xiaofeng et al., 2015; Brenton et al., 2005; Sørlie et al., 2003; Sotiriou et al., 2003) Possibility of pCR increase to over 70% may be reach also by double-HER2 blockade therapy (trastuzumab + lapatinib or trastuzumab + pertuzumab) additionally to the anthracycline/taxane-based treatment. (Coates et al., 2015) Surprisingly, pCR rate is higher in HR-/HER2+ in comparison with HR+/HER2+ BC tumors. (Cho, 2016; Gianni et al., 2014)

1.5.3.3 Basal subtype (triple negative breast cancer)

Tumors of basal subtypes are characterized by absence or low expression level of HR and HER2, high expression of basal markers (keratines 5, 6, 14 or 17 and epidermal growth factor receptor (EGFR)) and genes responsible for proliferation in cells. It is also usually associated low breast cancer genes 1 (BRCA1) expression, TP53 mutation and probability of grade 3. The primary tumors are usually larger than ones in other groups and show very quick growth, too. (Xiaofeng et al., 2015; O'Brien et al., 2010; Abd et al., 2005; Paik et al., 2004; Sotiriou et al., 2003; Sørlie et al., 2001) Basal-like tumors have the worst prognosis from all BC types, on the contrary Luminal A have the longest life expectancy. (Figure 7)

Set of triple negative breast cancer (TNBC) includes tumors of 6 main subtypes: two of them are basal-like, immunomodulatory, mesenchymal, mesenchymal and stem-like and luminal androgen receptor subtype. From these 6 subgroups, immunomodulatory subtype shows the best outcome while mesenchymal type shows the worst. (Prat et al., 2015; Lehmann et al., 2011)

Basal tumors are often associated with several important aspects. Younger age and earlier menarche, high waist-to-hip ratio, lack of breast-feeding belong to risk factors for this subtype. Lower disease-specific survival, higher risk of local and regional reccurence is associated with this tumor type. (Ho-Yen et al., 2012; Carey et al., 2006) Chemotherapy remains the only option of treatment. Patients achieving pCR rates after anthacyclines/taxanes therapy are accounted for 25-35% and have better outcome in comparison with those, who do not. (Cho, 2016; Xiaofeng et al., 2015; Liedtke et al., 2008; Brenton et al., 2005)

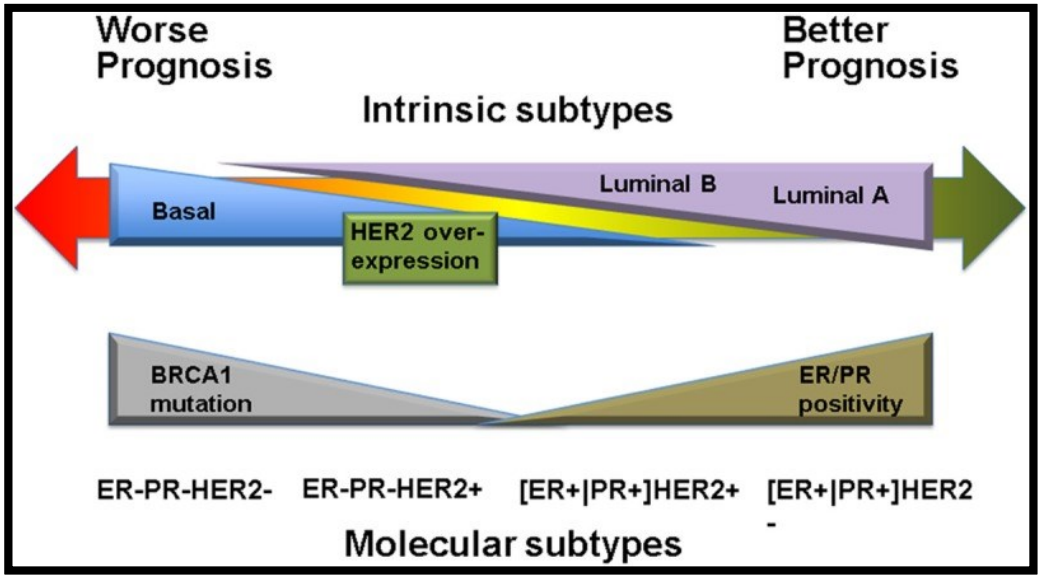

Figure 7: Patient outcome based on breast tumor various subtypes. ER, estrogen receptor; PR, progesterone receptor; HER2, Human Epidermal Growth Factor Receptor 2; BRCA1, Breast Cancer 1. (adapted from Xiaofeng et al., 2015)

1.5.4 Genes' contribution in breast cancer

Family history belongs to the most relevant risk factors in the case of BC manifestation. If an individual has a parent or a sibling with BC, relative risk for BC is doubled but if one has parent and a sibling with BC, relative risk quadruplicates. (Keeney et al., 2017) It is generally believed that up to 10% of all BC and 5% to 12% of this cancer appeared in women with age less than 45 years old and have been developed based on genetic predisposition. (Mehrgou and Akouchekian, 2016; Ottini et al., 2011) The others are usually caused by somatic, genetic and epigenetic alterations acquired during life. Whereas inherited mutations are usually as loss-of-function type, they affect genes linked to cell cycle checkpoint activation or DNA repair. These genes, which stimulate cell growth, survival and division, are altered in the terms of amplifications, deletions, rearrangements and gain-of-function mutation, epigenetic deregulation and micro RNAs and may contribute in abnormal genes expression which can result in BC development. (Lee and Muller, 2010; Stephens et al., 2009; Croce, 2009; Esteller, 2007)

The occurrence of germline mutations is usually rare. Frequencies of these heritable traits are different. The most important of hereditary mutations are found in breast cancer (BRCA), TP53, PTEN, Serine/Threonine Kinase 11 (STK11), Partner and

Localizer of BRCA2 (PALB2), E-cadherin (CDH1), Ataxia-Telangiesctasia mutated (ATM) or Checkpoint Kinase 2 (CHEK2) genes. (Figure 8) Although a degree of their penetrance can vary between high and low, lifetime risk of BC manifestation with these susceptibility genes is estimated to be 15-65%. (Kwong et al., 2016; Shiovitz and Korde, 2015; Apostolou and Fostira, 2013) (Table 4)

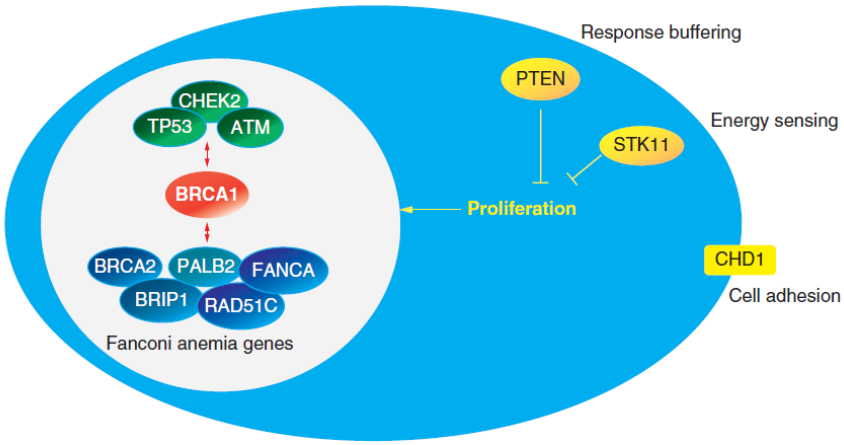

Figure 8: Representation of breast cancer predisposition genes. CHEK2, Checkpoint Kinase 2; ATM, Ataxia-Telangiesctasia mutated; BRCA1, Breast Cancer 1; BRCA2, Breast Cancer 2, PALB2, Partner and Localizer of BRCA2; FANCA, Fanconi anemia complementation group A; BRIP1, BRCA1 interacting protein 1; RAD51C, RAD51 homolog C. (adapted from O'Donnell et al., 2018)

On the contrary, acquired alterations result from a multistep process and include mainly genetic (such as copy number variations, etc.) and epigenetic (such as promoter methylation, etc.) changes. These modifications affect crucial cellular systems containing DNA repair, control of cell cycles mechanisms, adherence, differentiation, or normal cells metamorphosis into their malignant counterparts. Both proto-oncogenes' activation (such as phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), c-myc (MYC), etc.) or tumor suppressor genes (such as TP53, BRCA1, etc.) inactivation are partly responsible for cells transformation and also marvellous breast cancer heterogeneity. Somatic alterations have been usually associated with specific tumor characteristics and can be used as targets for personalized cancer therapy. (Ottini et al., 2011)

Table 4: Detailed description and characteristics of genes whose mutations contribute to BC manifestation.

Gene	Gene name	Protein name	Mutations outcome	Relative BC risk	Reference
BRCA1	Breast Cancer susceptibility gene 1	BRCA1	Microcephaly and grown disorder, Ovarian cancer	tenfold	Kurian et al., 2018; O'Donnell et al., 2018; Kwong et al., 2016; Mehrgou and Akouchekian, 2016; Mahdi et al., 2013; Stratton and Rahman, 2008
BRCA2	Breast Cancer susceptibility gene 2	BRCA2	Fanconi amemia type D1; Ovarian, prostate and pancreatic cancer	tenfold	Kurian et al., 2018; O'Donnell et al., 2018; Kwong et al., 2016; Mehrgou and Akouchekian, 2016; Apostolou and Fostira, 2013; Mahdi et al., 2013; Stratton and Rahman, 2008
TP53	tumor protein 53	P53	Li-Fraumeni syndrome; sarcomas, leukemia, brain tumors, adrenocortical carcinoma, lung cancer	At least 10-fold (18 – 60-fold)	O'Donnell et al., 2018; Kwong et al., 2016; Wang et al., 2013; Mahdi et al., 2013; Vousden and Ryan, 2009
PTEN	Phosphate and Tensin homolog	PTEN	Cowden syndrome or PTEN hamartoma tumor syndrome; Thyroid and endometrial cancer	At least 5-fold	Kurian et al., 2018; O'Donnell et al., 2018; Mahdi et al., 2013; Apostolou and Fostira, 2013; Tan et al., 2012; Pilarski, 2009
PALB2	Partner And Localizer Of BRCA2	PALB2	Pancreatic cancer	3 to 5-fold	Kurian et al., 2018; O'Donnell et al., 2018; Kwong et al., 2016; Slater et al., 2010

CHEK2	Checkpoint kinase 2	CHK2	Li-Fraumeni and Cowden syndrome; colorectal and bladder cancer	2 to 3-fold	Kurian et al., 2018; O'Donnell et al., 2018; Apostolou and Papasotiriou, 2017; Stracker et al., 2009
ATM	Ataxia Telangiectasia Mutated	ATM	Ataxia telangiectasia syndrome, ovarian cancer	2 to 3-fold	Kurian et al., 2018; O'Donnell et al., 2018; Choi and Kurzrock, 2016; Mahdi et al., 2013; Thompson et al., 2005)
STK11	Serine/threonine kinase 11	STK11	Peutz-Jeghers syndrome; ovarian, cervical, uterine, testicular, small bowel, and colon cancer	At least 5-fold	Jiang et al., 2019; Kurian et al., 2018; O'Donnell et al., 2018; Apostolou and Fostira, 2013; van Lier et al., 2010; Beggs et al., 2010
CDH1	Cadherin 1	epithelial cadherin / E-cadherin	Hereditary diffuse gastric cancer, colorectal cancer	5-fold	Lo, et al., 2019; Kurian et al., 2018; O'Donnell et al., 2018; Dossus and Benusiglio, 2015; Rakha et al., 2013; Apostolou and Fostira, 2013

1.5.5 Treatment selection and drug resistance strategies for different breast cancer types

Therapy choice belongs to critical parts of BC treatment process. Although wide platform of therapeutics is available for treatment of this diagnosis, picking out the most appropriate drug requires detailed examination of target tumor features because of BC's highly heterogeneous character. Key role in the selection process plays immunohistochemical analysis of HR and HER2 status which divides this disease into 3 basic groups as HR+, HER2+ and TNBC. (Kadam and Chaun, 2016; Tang et al., 2016; Tomao et al., 2015)

Despite correct initial treatment decision, development of drug resistance is not rare phenomenon in BC patients. It has been concluded disease resistance is hardly specified to one drug type. Instead, non-responding and progressive tumors may behave independently regardless therapy received, such as both chemo and monoclonal - antibody resistant. (Bianco and Gévry, 2012; Chen and Sikic, 2012; Wang et al., 2012; Vu and Claret, 2012; Musgrove and Sutherland, 2009)

1.5.5.1 Luminal types of breast cancer

Most of the BC patients have been included in HR+ HER2- group with often good clinical outcome. (Kumar et al., 2018; Viale et al., 2015; Prat et al., 2015) Recommended treatment strategies within this group are slightly different. Patients with rapidly progressive disease and key organs thread have often received chemotherapy to disease stabilization or it can be terminated from other reason (unbearable toxicity, as best as possible therapeutics effect achievement). Indeed, both BC patients with slowly progressive disease and after chemotherapy regimens have usually received endocrine therapy. (Novotny et al., 2016; Tang et al., 2016) Mechanism of action of these drugs type can differ. (Figure 9) Therapeutics used in this BC types are usually able to block ESR and estrogen binding (tamoxifen, toremifene), interfere with ESR synthesis (fulvestrant) or inhibit estrogen synthesis (anastrozole, letrozole, exemestane) and are also known as selective ESR antagonists/response modulators, ESR expression modulators/downregulators and aromatase inhibitors, respectively (Figure 9). Other strategy can represent ovary influence to stop them estrogen production (goserelin, leuprolide, triptorelin).

Ovarian function can be restored after stop taking medication. (American Cancer Society, 2017; Breastcancer.org, 2017)

Despite great success of endocrine therapy in BC patients' outcomes, usually up to 30% cases appeared to be resistant. (Tang et al., 2016) Mechanisms of this phenomenon need to be understood for better treatment effect achievement. It has been generally believed this behaviour of disease is caused by *de novo* or acquired resistance where both genetic and epigenetic changes contribute to tumor independent performance regardless of one and/or several therapies received. (Nass and Kalinski, 2015; Merenbakh-Lamin et al., 2013; Bianco and Gévry, 2012)

Different mechanisms may take role in the case of non-responding therapy status occurs in BC patients. Several mutation types have been revealed in estrogen receptor alpha (ESR1) gene and have been found to play key role in resistance contribution. One of these known genetics modifications, D538G or Y537S/C/N, causes conformation modification of ESR1 ligand binding domain which results in not only tamoxifen lower affinity and ineffectiveness but also cell proliferation and tumor progression without hormone stimulation. Other known dangerous event based on fusion has been discovered. Patients with ESR1-CCDC170 mutation identified demonstrated more aggressive progression of tumor and higher endocrine treatment resistance. (Lei et al., 2019; Tang et al., 2016; Li et al., 2013) Different mechanism can be based on alternative pathways evolution which usually does not require genetic contribution. Tamoxifen resistance may be achieved by adaptive responses development for tamoxifen induced estrogen signalling blockage bypass. Examples of this situation are tumor suppressors' downregulation (PTEN) or drug resistance drivers' upregulation (protein kinase B (AKT)). (Figure 9) Other known resistance mechanism is active drug concentration reduction through metabolism in cells. (Tang et al., 2016; Nass and Kalinski, 2015; Raha et al., 2011)

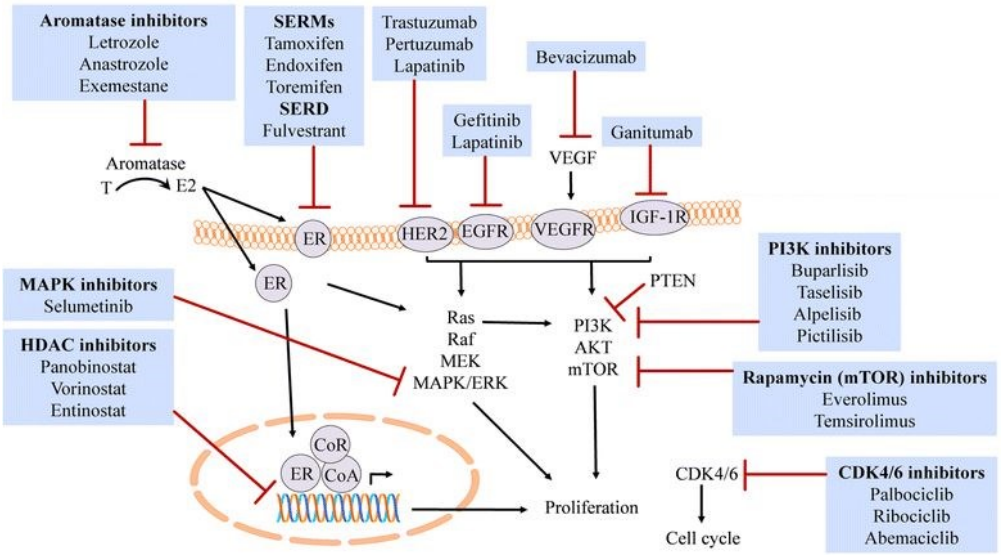

Figure 9: Schematic presentation of drugs and their signalling target used in endocrine BC treatment. CDK, cyclin-dependent kinase, E2, oestradiol-17 beta, EGFR, epidermal growth factor receptor, ER, oestrogen receptor, HDAC, histone deacetylase, HER2, human epidermal growth factor receptor, IGF-1R, insulin-like growth factor-1 receptor, MAPK, mitogen-activated protein kinase, mTOR, mammalian target of rapamycin, PI3K, phosphoinositide-3-kinase, SERD, selective oestrogen receptor degrader, SERM, selective estrogen receptor modulator, T, testosterone, VEGF, vascular endothelial growth factor, VEGFR, VEGF receptor. (adapted from Selli et al., 2016)

1.5.5.2 Human Epidermal Growth Factor Receptor 2 - enriched subtype

Despite relatively poor prognosis of BC patients with HER2 overexpression, these tumors usually respond well to HER2-receptor targeting therapies. (Cho, 2016) Trastuzumab, as humanized monoclonal antibody, targets HER2 extracellular domain and has been connected to BC patients' survival enhancement. Since more than half of the treated patients with trastuzumab either develop relapse or do not respond, essential part of resistance mechanisms have been found out and have led to discover effective alternatives to current treatment options. (Butti et al., 2018) Detailed research has described several mechanisms such as changed drug binding property, downstream pathways modification or alteration HER2 status have been revealed to significantly contribute in BC patients *de novo* or acquired resistance. Altered binding property of trastuzumab can be achieved by both enzymatic cleavage of target site or epitope masking where mucin 4 (MUC4) and CD44/hyaluronan polymer activation has been associated with considerable reduced binding efficiency. Other defends of cancer cells can be modified regulation of downstream pathways. Elevated heterodimer formation or other ErbB family members activation can results from trastuzumab induced signalling

blockage bypass. Another mechanism includes receptor status change where lack of HER2 can cause trastuzumab inefficiency (Figure 9). (Tang et al., 2016; Gelmon et al., 2015; von Minckwitz et al., 2011; van de Ven et al., 2011)

1.5.5.3 Triple negative type of breast cancer

Despite TNBC is generally characterized based on negative expression of ESR, PGR or HER2, it has been identified at least 7 different TNBC subtypes characterized by particular biology features (Figure 10). Since no targeted treatment has been officially accepted for this diseases type treatment, traditional cytotoxic chemotherapeutics, anthracyclines and taxanes have been commonly used. Resistance occurred event in the TNBC patients is not rare, emergence of novel treatment strategies is essential. (Tang et al., 2016; Novotny et al., 2016; Abramson et al., 2014; Mayer et al., 2014)

Newly, immunotherapy regimens have been officially approved for TNBC patients. If tumors show Programmed death-ligand 1 (PD-L1) positivity of at least 1% on the tumor infiltrating immune cell, response was observed. (Bayraktar et al., 2019; Mina et al., 2019; The Cancer Genome Atlas) Immunotherapy opens a completely new bright field of the TNBC treatment. It has been also reported objective response was observed in the pre/treated chemoresistant TNBC patient. (Ponomarenko et al. 2020) As expected, the tailoring of the patients into the grous of immunotherapy receivers and non-receivers is quite challenging, whereby there are no strict rules how to decide in this very specific TNBC cases.

Traditional and new therapeutics have been examined alone or in combination with other drugs. Platinum agents (cisplatin, carboplatin) belong to the conventional medications and cause DNA crosslink strand breaks. Patients with this medication combination have reached higher pCR rate without anthracyclines in transformation-related protein 63 (p63) positive tumor patients. Several studies have confirmed favourable pCR rate of cisplatin in TNBC patients. (Silver et al., 2010; Byrski et al., 2009; Rocca et al., 2008; Leong et al, 2007)

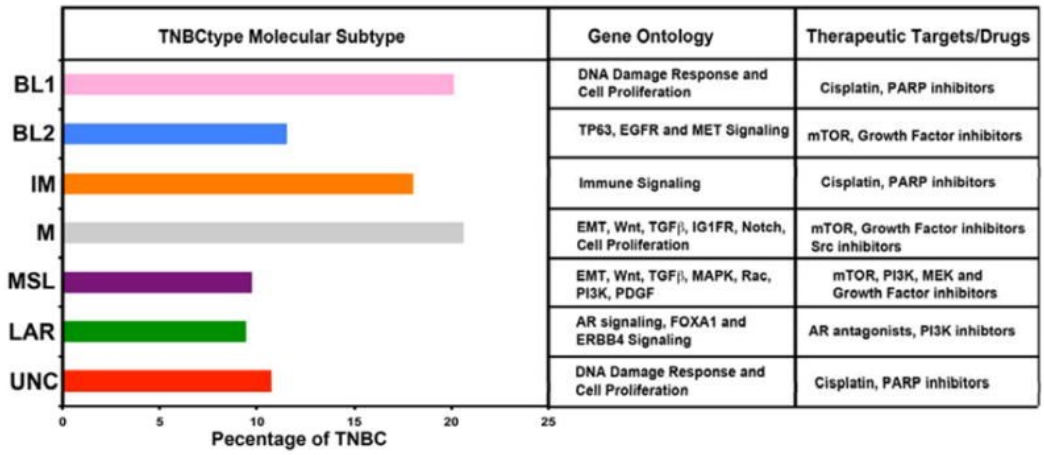

Figure 10: TNBC subtypes distribution from The Cancer Genome Atlas (TCGA) with enriched gene ontology and potential therapeutic targets. TNBC, triple-negative breast cancer; BL1, basal-like subtype 1; BL2, basal-like subtype 2; IM, immunomodulatory subtype; M, mesenchymal subtype; MSL, mesenchymal stem cell-like subtype; LAR, luminal subtype expressing androgen receptor; PARP, poly-AD-ribose polymerase; TP63, tumor protein 63; EGFR, epidermal growth factor receptor; MET, MET proto-oncogene receptor tyrosine kinase; mTOR, mammalian target of rapamycin; EMT, epithelial-mesenchymal transition; Wnt, Wnt proto-oncogene; TFGβ, transforming growth factor β; IG1FR, insulin-like growth factor 1 receptor; MAPK, mitogen-activated protein kinase; Rac, ras-related family of proteins; PI3K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; MEK, mitogen-activated protein kinase kinase; FOXA1, forkhead box protein A1; ERBB4, v-erb-a erythroblastic viral oncogene homolog 4; AR, androgen receptor; UNC, unclassified. (adapted from Abramson et al., 2015)

Poly-ADP ribose polymerase (PARP) inhibitors have seemed to become another promising approach that exhibited favourable results in BC patients carried BRCA ½ mutation. (Robson et al., 2017; Telli et al., 2013) Target of this therapeutics is PARP enzyme which is essential in DNA repair pathways.

Another possible treatment strategy in TNBC has seemed to be phosphatidylinositol-3-kinase (PI3K) pathway inhibition. It has been observed TNBC cells sensitization to DNA-damaging agents after PI3K inhibition. Combination of PI3K inhibitors which interact with homologous recombination complex followed by DNA-damaging agents has been believed to become efficient approach. (Mayer et al., 2014; Ibrahim et al., 2012; Juvekar et al, 2012; Wong et al., 2010)

TNBC is heterogeneous disease which should not be treated uniformly. A therapy setting is one of the most challenging tasks in this diagnosis. Although one of the strategies is potential targets identification, TNBC biology may allow the disease multiple mechanisms development to survive and spread. Events of selective

inhibition one pathway followed by compensatory pathway activation are not rare. Approach using two or more targeted medications has seemed to have more success than single one. (Figure 10) (Novotny et al., 2016; Mayer et al., 2014)

Currently, if applicable, neoadjuvant therapy in the form of combined anthracyclines and taxanes regimens is usually prescribed to TNBC patients. Results of this medication administration have revealed at least 40% of patients do not reach pCR. These patients have usually recurrence within upcoming 36 months. Approximately 30% of patients achieve pCR after neoadjuvant treatment. After neadjuvant chemotherapy a surgical removal of the resting tumor residuum is planned. In the case there is no clinical evidence of metastatic disease after tumor excision; patients stay in the observation without any other treatment. Although it is generally believed TNBC patients who have pCR achievement show favourable long-term outcome, applying no additional adjuvant treatment strategy has not been recommended by latest research data. However there are some similarities in the character of pre- and post- treatment tumor. Suitable post-neoadjuvant therapy is needed and may be personalized according to individual patients‘tumors subtypes. (Mayer et al., 2014; Balko et al., 2012; Guarneri et al., 2009; Jones et al., 2009)

1.6 Biomarkers

Personalized medicine, in the oncology field, relies on individual differences in patients gained from biomarkers screening. It also provides opportunities and impacts included in four aspects, known as P4: Preventive, Predictive, Personalized, and Participatory. (Kalia, 2013; Cho et al., 2012) Preventive represents ability to prognosticate disease risk or presence before any clinical symptoms appear. Role of predictive part of the P4 group is to help with optimal therapy choice for defined subgroup of patients. (Cho et al., 2012). Personalized section is focused on targets with positive effects on disease following by safe and effective development treatment based in this information. Participatory part is interested in patients‘treatment adherence. Useful effects and minimal side effects can result in willingness of taking patients’ drugs more responsibly. (Cho et al., 2012)

If speaking about the personalized medicine, we have to define the term precision medicine as well, as they both relate together. Precicion medicine is the medicine part where all of the treatment decisions are made based on the precise knowledge of the patients' genom, in tumor biology - tumor genom in addition. A big increase of next generation sequencing applicability in daily clinical routine has enabled to combine the knowledge of the genotypes and phenotypes into an evidence - based decision, which is valued pretty much in the current oncology approach.

Biomarkers sources are reliable and accurate measurements that indicate a normal biological process, a pathogenic process, or a pharmacological response to a therapeutic intervention. It includes clarifying parameters such as molecular and cellular measures from biological fluids (blood, plasma, serum, etc.), histopathological analysis of tissue samples or measurements by magnetic resonance images. (Cho et al., 2012) Biomarkers can be divided into standard and molecular biomarkers. Standard biomarkers have been already established into routine analysis which includes morphologic and functional characteristics (histology and grading of tumor, invasion presence, etc.). Molecular biomarkers are consisted of compounds and molecules that are part of the genome, epigenome, transctiptome, etc. Their classification is then depending on specific characterisation (nucleic acids, proteins, hormones, etc.). Molecular markers can be divided into prognostic, predictive or diagnostic according to their function. Diagnostic biomarkers'occurence is associated with particular diagnosis and contribute to accurate diagnosis determination, prognostic biomarkers are connected with survival prediction, and role of predictive biomarkers is to forecast a probability of disease response to specific treatment. (Foretova et al., 2014; Williams and Stoeber, 2012; Ong et al., 2012; Reis-Filho and Pusztai, 2011)

In the context of cancer, molecular alterations that have been used as biomarkers occur in DNA (gene replication, rearrangements/translocations, point mutations/deletions or insertions), RNA (changes in transcription and post-transcriptional modifications) and protein (changes in translation and post-translational modifications). Newer approaches are also using miRNAs which are small nucleotides (19-25bp) and take an attention by their regulation activity. (Goldstein et al., 2013; Demidyuk et al., 2013; Sethi et al., 2013; Ahmed et al.,

2012; Kong et al., 2012; Bao et al., 2011) As next, exosomes and circulating free DNA can be tested in the circulating body liquids, too.

1.6.1 Diagnostic biomarkers

One of the requirements for molecules to be used in cancer diagnostic processes is their presence in various clinical specimens such as blood, fine needle aspirates or fresh frozen and paraffin embedded tumor tissue. Some of these diagnostic biomarkers can also act as targets for therapies. Number of this cancer biomarker portfolio is still growing. Molecules which are proven to be useful have already been incorporated in the clinical patients' management. (Gall et al., 2013; Sethi et al., 2013; Ali et al., 2012; Liu et al., 2012; Liang et al., 2007)

Diagnostic biomarkers also contribute to revelation of cancers of unknown primary origin (CUPs). It is very important because almost one of three cancer patients have already distant metastasis at the time of diagnosis. These types represent diagnostic of 150000 new cases of this type in the USA and the European Union. CUPs are found to be related to poor prognosis and non-selective empirical therapy treatment.

Protein serum - based markers have been already introduced to the clinical practice and contribute to the determination of proper diagnosis, but most of them are not often specific enough. For example carcinoembryonic antigen (CEA) levels are test in gastrointerstinal tumors of epithelial origins, prostate specific antigen (PSA) is a leading marker in the diagnosis and treatment of prostate cancer in men, increased cancer antigen 125 (CA-125) levels may point on ovarian cancer in women. (Tomuleasa et al., 2017; tothill et al., 2013; Handorf et al., 2013)

Performing molecular profiling of CUPs tumors is mainly based on the DNA, messenger RNA (mRNA), microRNA or epigenetic changes evaluation. Commercially available tests (often works based on mRNA and microRNA evaluation) are dependent on RNA quality and much more expensive in comparison with immunohistochemistry. Due to the CUPs diagnosis difficulty, both immunohistochemistry and molecular methods should be used to reveal correct diagnosis of diseases. (Tomuleasa et al., 2017; Oien and Dennis, 2012; Economopoulou et al., 2015; Golfinopoulos et al., 2009; Lazaridis et al., 2008)

1.6.2 Prognostic biomarkers

Information on clinical outcomes of patients (overall survival, recurrence-free survival), independently of treatment, are provided based on prognostic biomarkers. A clinical useful prognostic biomarker must be an independent, significant factor that is easy to be determined and interpreted and that has some therapeutic impact. It can be used for patients' treatment selection without treatment response forecast. (Cho et al., 2012; Ong et al., 2012; Buyse et al., 2011) However, therapy progress, evaluation of the tumor stage and its malignancy potencial or disease remission prognosis can be monitored through levels and concentrations of the prognostic biomarkers, too. (Nalejska et al., 2014) In the case of BC, adjuvant systemic treatment choice is usually based on nodal status, tumor size, tumor type/grade, lymphatic and vascular invasion, age, and ethnicity. Patients are classified into 5 molecular subtypes according to immunohistochemical expression detection of ESR, PGR, HER2 or mindbomb E3 ubiquitin protein ligase 1 (MIB1) antibody (Cheuk et al., 2017; Brouckaert et al., 2012; Cho et al., 2012)

Prognostic biomarkers are evaluated according to specific tumor types by proven presence of single nucleotide polymorphisms, specific mRNA molecules or CTC, mutations, DNA methylation changes or altered gene expression. (Nalejska et al., 2014)

Some of the genes responsible for normal cells growth can mutate which may results in increased risk of cancer diseases in individuals. These abnormal genes can be transferred from one generation to the next together with higher risk of developing cancer although majority of cancer patients have no family history of this disease.

Genetic testing is recommended to reveal possible mutation presence based on the tumor type. Abnormal BRCA1, Breast Cancer 2 (BRCA2) and PALB2 genes contribute higher than average risk of developing breast and/or ovarian cancer. Changes in these genes may account for up to 10% of all breast cancers. (Nalejska et al., 2014; Genetic testing: Breastcancer.org). In addition to these mutation analyses, expression profiles of breast tumors are an important part of tumor outcome determination. Based on all the information, BC tumors are then divided

into several subgroups with different prognosis and treatment options. (Foretova et al., 2014)

1.6.3 Predictive biomarkers

Information about probability to react on administered treatment can be helpful in therapeutics regimens decision processes. It could be provided by predictive biomarkers. Screening of tumor specific features is important for several purposes, such as, reducing unnecessary treatment, avoiding toxic effects of therapeutics regimens or decreasing morbidity. These markers are supposed to reach overall health care cost reduction and may enhance quality of patients' life. (Nalejska et al., 2014; Cho et al., 2012; Ong et al., 2012; Voon and Kong, 2011; Duffy et al., 2011; Walther, 2009; Suter et al., 2007)

BC belongs to diseases that have been cured by first monoclonal antibody known as trastuzumab. It belongs to target therapy regimens because this humanized monoclonal antibody is directed against extracellular domain of HER2. Expression of HER2 is a critical indicator of multiple downstream pathways activations, which are required for the uncontrolled proliferation of cancer cells. (Foretova et al., 2014; Vu and Claret, 2012) It has been found out that HER2 overexpression (tumors known as HER2-positive subtypes) is present in 20-30% of breast cancers. These tumor subtypes are associated with lower disease-free survival and overall survival rates in comparison with other subtypes of BC. (Vu and Claret, 2012; Browne et al., 2009)

Numbers of clinically valuable predictive biomarkers are still growing. Changes in genes which are reflecting therapy responses are likely to be caused by point mutations and chromosomal aberration. One of the most studied gene alterations, screening mainly in the cases of CRC, are mutations in KRAS gene. Presence of this type of mutation is the most common in codons 12 and 13 and less common in 61 and 146. (Nalejska et al., 2014; Loupakis et al., 2009) Results of this screening are helpful during therapy decision process for monoclonal antibody treatment (cetuximab) against EGFR extracellular domain. (Figure 11) KRAS is an important player in several signalling pathways in cells and its mutations lead to abnormal

growth, proliferation and differentiation actions. (Nalejska et al., 2014; Ong et al., 2012; Walther, 2009)

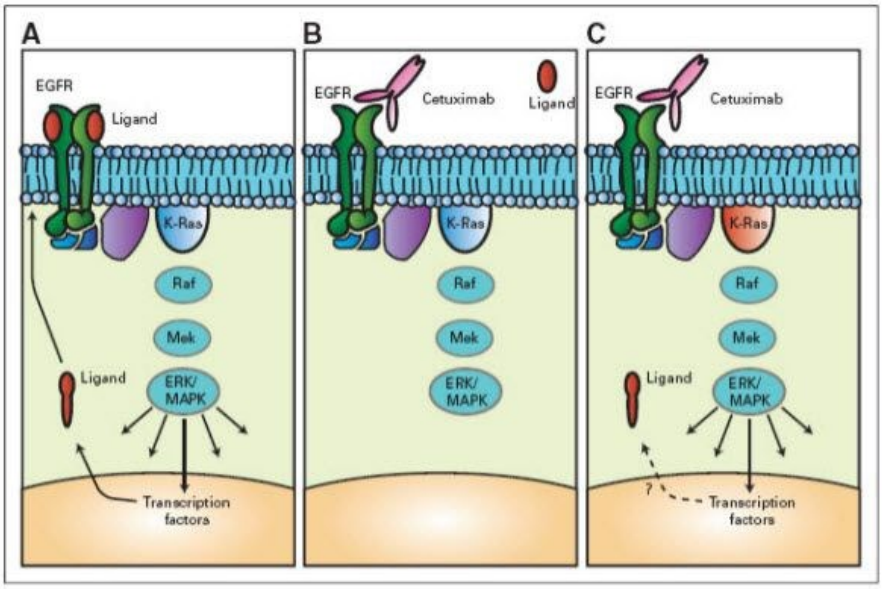

Figure 11: Representation of ligand binding to the EGFR and it triggers signaling through the Ras/MAPK pathway to multiple targets that may regulate ligand levels (A). Monoclonal antibodies to EGFR (cetuximab and panitumumab), prevent ligand binding and deactivate EGFR signaling (B). Mutation in KRAS can result in dysregulation of the MAPK pathway and downstream signaling in the absence of ligand-dependent receptor activation (C). MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor. (adapted from Eng, 2010)

1.7 Liquid biopsy

Additional testing to classical biopsies has been intensively discussed for decades. In ideal situation, the new test should be an easily approachable alternative to the relatively complicated process of tissue biopsy. Taking this fact into account, one would consider examination of bodily fluids. This is how the liquid biopsy (LB) era officially started.

Nowadays, information obtained from classical tissue biopsy is to be compared and completed by LB, which includes testing of CTC and ctDNA in the most of the cases. Additionally, testing of circulating exosomes or/ and microRNA may be added. The term LB is used for sampling method of non-solid biological tissue which usually includes blood, salina, urine, pleural effusion, cerebrospinal fluid and other body fluids. Patients' blood, as LB, is widely used in oncology. (Figure 12) LB is a minimally invasive or non-invasive alternative to surgical biopsies which provide data on tumor cells from simple blood sample. LB is used for patients'

stratification, screening, tumor development and real-time treatment response monitoring and also to detect minimal residual disease after surgery or in era of possible recurrence. (Brock et al., 2015; Crowley et al., 2013)

Patients with metastasis have often micrometastasis or macrometastasis in their organs but not all of them are always clinically detectable. These tumor foci often represent risk of disease progression due to several reasons. Direct biopsy of tumor or metastatic tissue is not always possible due to invasiveness or hard access. (Table 5) Significant cost of these biopsy examinations is one of the arguments which prevent repeating and following tumor changes through time. For completion of diagnosis sufficient amount of tumor sample is necessary to obtain which is not always possible. Classical biopsies analysis is not usually achievable without conservation techniques which often lead to false positive genetic tests. (Brock et al., 2015; Gkountela et al., 2016)

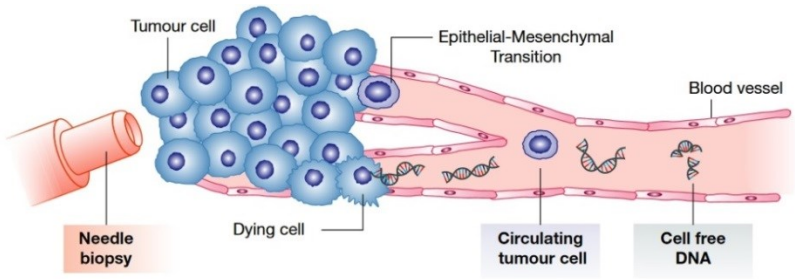

Figure 12: Schematic representation of liquid biopsy – withdrawal from blood veins of cancer patient. (adapted and modified from Wyatt and Gleave, 2015)

There is also growing evidence on negative biopsy impact regarding mechanical tumor integrity. Cancer cells are known for their uncontrolled growth and proliferation which is connected to lack of cohesiveness. The cancer cells are not coherent enough to resist dislodging biopsy, surgical intervention or needle aspiration and result in migration and colonization. This theory is supported by case studies where patients developed cancer or exhibited tumor cells presence in their bloodstream after diagnostic biopsy or surgery in various cancer types. (Alieva et al., 2018; Yu et al., 2018; Kaigorodova et al., 2017; Marshall and King, 2016; Shyamala et al., 2014; Kuo et al., 2012)

Samples from classical biopsies hardly reflect cancerous population of individual patients. (Table 5) Tumors and metastasis are usually consisted of cells with

heterogeneous profiles and evolutionary process of metastasis often forms new metastatic sub-clones with high heterogeneity degree whose results from critical differences between both primary tumor and metastasis and also metastasis themself. Despite these facts, patients are often treated based on information obtained from their primary tumors. (Aaltonen et al., 2017; Turajlic and Swanton, 2016; Gkountela et al., 2016; Valastyan and Weinberg, 2011)

Table 5: Comparison of biopsy, CTC and cell-free tumor DNA characteristics in patients with solid tumors. (according to Schaffner et al., 2020)

	Metastatic biopsy	**CTC**	**Cell-free tumor DNA**
Logical / practical considerations	Invasive, more difficult to obtain	Easy to obtain	Easy to obtain
	Expensive (interventional radiology, pathology)	Inexpensive (blood draw)	Inexpensive (blood draw)
Pre-analytical considerations	Serial testing less feasible More difficult to control (sample processing, such as time to fixation, may cause artifacts)	Easy serial testing Easier to control: pre-specified fixative, anticoagulant in collection tube	Easy serial testing Easier to control (pre-specified fixative, anticoagulant in collection tube)
Sensitivity	Abundance of cells (10^6-10^8 cells per biopsy)	Low cells number (~ 1-1000 cells per 7.5 ml whole blood)	Low nucleic acid content (10^2-10^4 ctDNA copies/ml whole blood)
Phenotypic assays	Immunohistochemistry (e.g. ESR, PGR, HER2)	Immunohistochemistry (e.g. ESR, PGR, HER2)	N/A
Genomic assays	Comprehensive NGS feasible (whole genome, exome, transcriptome, copy number analyses)	NGS feasible for candidate genes (n = 10-100) depending on volume of CTC/nucleic acid content	NGS feasible for candidate genes (n = 10-100) depending on volume of nucleic acid content
Biologic considerations	Only represents one tumor site	May represent more comprehensive tumor assessment	May represent more comprehensive tumor assessment
	Represents biology of tissue-based cancer at that site	May not represent biology of “tissue-based” cancer	Unknown
	Represents “live” cancer cells	Represents “live” cancer cells	Represents apoptotic cells or secreted exosomes

ESR, estroger receptor; PGR, progesterone receptor; HER2, Human Epidermal Growth Factor Receptor 2; CTC, circulating tumor cells; NGS, next-generation sequencing; N/A,not applicable

In general, blood as LB is an attractive concept to overcome limitations of classical biopsy and also allows measuring other human blood components status. CTC and

ctDNA are widely used as circulating blood-based biomarkers in oncology. Both of these analytes can significantly contribute to improve cancer diagnosis, therapy guidance or disease monitoring. Despite both of them are easy to obtain from patient, their frequency is very low so proper characterisation is challenging. (Schaffner et al., 2020; Bidard, 2019; Neumann et al., 2018; Calabuig-Fariñas et al., 2016) (Table 5) Moreover, their lifetime has been found to be similar. Half-life of single CTC, CTC cluster and cell-free tumor DNA is between 0.5 – 2.4 hod, 6-10 min and 16 min - 2.5 h, respectively. (Bronkhorst et al., 2019; Kustanovich et al., 2019; Lozar et al., 2019; Ye et al., 2019; Chen et al., 2017)

One of the most important difference between these two markers is their origin. CTC are shed from tumor tissue and most of them undergo apoptosis as a result of adhesion to the extracellular martix loss, hemodynamic shear forces, body immune system attack or drugs target. On the other hand, ctDNA is derived from apoptotic and necrotic tumor cells which released their DNA into blood circulation. CtDNA is a small fraction of cell-free DNA which is non-cancerous origin and its detection relies on tumor-specific detection of cell-free DNA. In general, significant deficiencies in cell-free DNA approach are (1) the lack of defining details of tumor heterogeneity; (2) only relative copy number rather than absolute one can be obtained; (3) establishing *in vivo* and *in vitro* models (such as cells lines, organoids or animal xenograft modelling) is not possible; and (4) lack of transcriptomic or proteomic assays. (Schaffner et al., 2020; Ye et al., 2019)

Thus, blood withdrawal, included in sampling known as LB, is less invasive for patients in comparison with classical biopsy and can be performed during medical examination in every ambulance. (Figure 12) Information from patients' blood is gained using biomarkers presence, quantity and characteristics. There are several assays types which can be implemented for complex characterisation of tumor disease. (Table 6)

Table 6: Schematic overview of assays types and value of obtained information. (taken and adapted from Schaffner et al., 2020)

Type of analysis	Information
Enumeration	Prognostic value (OS, PFS), cancer recurrence detection, real-time monitoring of tumor sensitivity and acquired resistance, algorithm for treatment to huide the use of marker levels over the treatment course
Gene expression	Mechanism underlying tumor metastasis understanding, identification of gene markers (disease progression and treatment response prediction) and tissue of origin
Mutation assays	Recognition of actionable targets and acquired resistance via acquired mutations
Sequencing	Novel targets for potentional therapies identification

OS, overall survival; PFS, progression-free survival

LB's investigation allows us to evaluate tumor-specific genetic alterations, to influence the therapy and to reflect resistance development. One of the most primising biomarkers is CTC which enable scientists a real-time diseases monitoring. Their obtaining is minimally invasive for patients and they can be widely used for microscopy or both phenotypic and genomic assays. (Table 5) They offer information obtaining directly from intact malignant cells. (Schaffner et al., 2020; Müller Bark et al., 2019; Soda et al., 2019; Bai and Shao, 2018; Buder et al., 2016; Brock et al., 2015)

1.8 Circulating tumor cells

Metastases are still the most common reason of death in the cases of oncological disease. New methods for real-time disease'monitoring have been developed nowadays. One of the most promising approaches uses CTC which may become indispensable in future. CTC are shed from tumor tissue, circulate through body of cancer patients and might proliferate and set up metastasis. (Figure 13) They belong to rare cell group placed in peripheral blood of cancer patients. They can be useful in real-time disease monitoring mainly due to their short half-life in circulation. CTC reflect actual diseases status and have been shown to be more accurate than biopsies. (Bulfoni et al., 2016; Lohr et al., 2014; Ni et al., 2013; Heitzer et al., 2013)

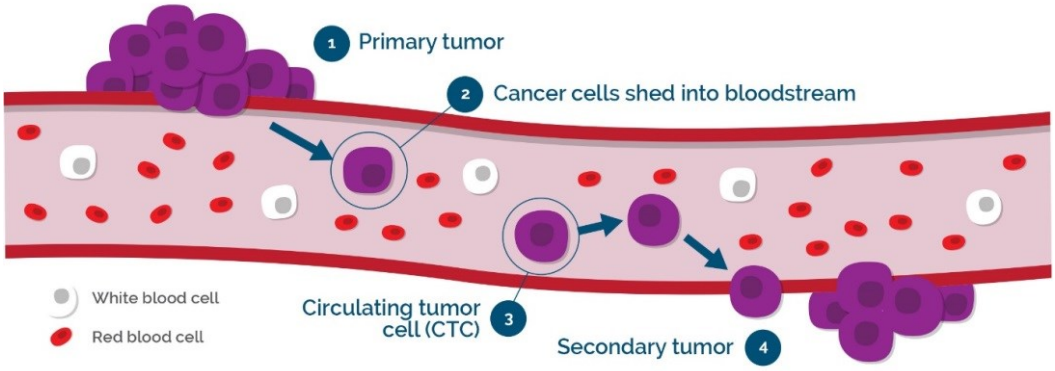

Figure 13: Schematic illustration of tumor cells spread through blood circulation. (adapted from *Circulating Tumor Cells VS Tumor Biopsies*, 2019)

CTC as biomarkers can offer several valuable information about cancer patients' tumor but the information depends very much on their separation process, detection and characterization. Although frequency of CTC in patients' peripheral blood is often very low, enrichment methods can be introduced for successful CTC separation.

1.8.1 Circulating tumor cells enrichment

Separation of CTC has been included in CTC characterization process to capture as much CTC as possible for following analysis. Heterogeneity of these cells has forced scientists to apply new methods to reach pure and representative CTC population typical for individual oncological patients. Nowadays, the most used enrichment methods are based on differences in physical and/or biological properties of CTC in comparison with other cells present in the blood. (Table 7)

Table 7: Schematic list and description of CTC enrichment methodologies. (taken and adapted from Bankó et al., 2019; Chen et al., 2017)

	Method	Advantage	Limitation
Biophysical properties	Size/deformability based filtration	Easy and fast method High throughput High CTC and CTC clusters capture efficacy Downstream analysis of enriched cells Label-free CTC Unmodified and viable cells Short enrichment time Less cost due to lack of labels	Loss of CTC with size equal to and smaller than pore size of filter CTC damage due to hemodynamic stress Flow pressure Possible interference with other cells (leucocytes) Validation issues (difference in size between CTC and cancer cell lines models)
	Density-gradient centrifugation	Easy to use High throughput	Loss of CTC with high density Low purity
	Electric property based dielectro-phoresis	High CTC viability	Loss of CTC with electrical property similar to leukocytes Low purity
Biological properties	Positive selection (antibody/ies-mediated isolation)	High purity Easy to scale up Automated systems Downstream analysis	Loss of CTC subpopulations including EMTed CTC, clusters, and CTC cloaked by blood cells (no universal CTC antigen have been identified) Tumor-specific assays may be required since CTC phenotype varies by cancer type and over disease progression and treatment course CTC bound to the surface of a device can cause difficulties in cell recovery

	Negative selection (depletion of blood cells)	Potential to enrich all CTC subpopulations Easy to use batch separation Label-free CTC Unbound CTC	Low purity

CTC, circulating tumor cells

1.8.1.1 Physical properties used for isolation of CTC

Isolation methods based on physical properties of CTC have become popular nowadays. They depend on characteristics such as size, deformability, density or electric charge which allow capturing of heterogeneous cells with various gene expression and protein profiles. (Table 7) Some of these methods enable to isolate viable CTC which may be helpful in their further functional characterization. (Ferreira et al., 2016; Zhang et al., 2016)

Physical parameter which is often used as typical for CTC is their size. It has been reported that the majority of CTC is usually bigger than blood cells in various cancer diagnosis, although it is dependent on cancer cell fitness, different stages of cell cycle, media composition or fixation process. Size is utilized in the size-based separations technologies where filters are used to remove the most of the blood cells from blood samples. It has been also observed that there is enormous size heterogeneity between CTC in a patient. CTC have sometimes irregular shape, irregular nuclear membrane, multiple nucleoli and also form aggregates - in these aggregates CTC are smaller than non-aggregated CTC. (Bobek and Kolostova, 2018; Zhang et al., 2016; Cho et al., 2012; Lecharpentier et al., 2011; Tan et al., 2010)

Deformability is another physical property used in the CTC separation technologies. Several studies have suggested connection between deformability, tumor cells and metastatic potential. In most of the studies higher deformability in tumor cells in comparison to nonmalignant cells has been observed. According to Osmulski et al., CTC isolated from castrate-resistant prostate cancer patients were about 7 times more adhesive and 3 times more deformable and softer than castrate-

sensitive specimens. (Vazquez et al., 2015; Osmulski et al., 2014; Zhang et al., 2012; Gossett et al., 2012)

Cancer cells can be enriched also based on their density which usually differs among cells present in blood stream. Based on different density a centrifugation process is followed by layers creation, layers are represented by cells with particular density typical for specific layers. It had been found that density of mononuclear cells and CTC is <1.077 g/ml and other blood cells and granulocytes is >1.077g/ml. This principle of CTC isolation is considered to be quick and simple but there is a risk of CTC loss by their aggregation. (Zhang et al., 2016; Rosenberg et al., 2002)

Dielectrophoresis uses cell separation approach based on electrical properties of CTC applying non-uniform electric field. Cells with diverse dielectric properties can be manipulated or transported or separated by dielectrophoresis. Although this method is relatively slow, it enables to isolate cells with high accuracy and precision. (Zhang et al., 2016; Ferreira et al., 2016; Harouaka et al., 2014)

1.8.1.2 Biological properties used for isolation of CTC

CTC enrichment based on biological characteristics of cells works usually based on affinity reactions. Antigens which are present on the target cells surface are captured by antibodies. There are two types of this immunologic selection, positive and negative. (Table 7) Positive selection procedure consists of pulling CTC out from other blood particles via antibodies against tumor-associated antigens (usually anti epithelial cellular adhesion molecule (EpCAM)). On the other hand, negative selection is removing other blood particles to "concentrate" CTC in resting specimen (usually anti-CD45 is applied). Although these types of separation may be highly specific and automatized, the newest studies are expressing concern about capture and analysis of heterogeneous CTC population in blood. Samples of cancer cells may undergo EMT and CTC of mesenchymal character are usually lost by applying anti-EpCAM approach. Moreover, cells with EpCAM expression has been also found in individuals with benign disease. (Gabriel et al., 2016; Ferreira et al., 2016; Pantel et al., 2012)

CellSearch® system is the only FDA (US Food and drug Administration) approved technology for CTC enrichment. Principles of this immunomagnetic enrichment are represented by ferrofluid particles with an EpCAM antibody which separate EpCAM positive cells from other blood particles. As next, immunostaining procedure to prove cytokeratin-8 (KRT8), cytokeratin-18 (KRT18) and cytokeratin-19 (KRT19) expression is applied together with fluorescent nuclear staining by 4',6-diamidino-2-phenylindole (DAPI) and lack of CD45 expression confirmation determined by negative CD45 staining. As the historically first accredited CTC separation system, it is used as a gold standard in comparison to other separation methods. (Su and Nieva, 2017)

AdnaTest® (Adnagen AG, Germany) is also using immunomagnetic separation for CTC enrichment and detection. Separation of CTC is provided by magnetic, antibody-coated beads. Unlike Cellsearch®, in AdnaTest® a cocktail of 3 various cancers type specific antibodies are used. Tests for BC, prostate, ovarian and colon cancer have been already prepared and tested. Detection of captured cells is based on multiplex PCR reactions, where an expression of tumor-associated markers is determined. (Ferreira et al., 2016)

These two methods, CellSearch® and AdnaTest® have already been tested in parallel resulting in positivity rate for CTC in metastatic CRC patients (33% patients positivity when using CellSearch® (≥3 CTC); 30% positivity when using the AdnaTest®; 50% if both assays were combined) but unfortunately 50% of patients were evaluated as negative despite of clinically detectable metastasis presence. (Gorges et al., 2016)

Other relatively flexible methods have been developed for CTC enrichment by magnetic separation. They are based on magnetic beads connected with antibodies. Magnetic cell sorter (MACS) captures cells labelled with magnetic nanoparticles conjugated to antibodies for purposes of cell separation, enrichment and depletion. (Giordano et al., 2012; Pluim et al., 2012) Similarly, Magsweeper® technology is able to use unfractionated blood to isolate viable cells, based on immunomagnetic strategy. (Powell et al., 2012)

1.8.2 Circulating tumor cells identification

Some of the CTC enrichment methods mentioned earlier applies a CTC determination step immediately after enrichment. Enumeration and characterization of CTC have found to represent a critical step of monitoring. Various technologies have been developed for their analysis but the most used ones are based on simple principles.

1.8.2.1 Evaluation of enriched cells

Detection of CTC based on cytopathologic characteristics is one of the direct analysis methods. It is relatively fast and offers specificity, simplicity and low cost. It may bring significant value in identification of oncological patients with high recurrence or metastasis risk and invasive tumors. It has been also reported that experienced pathologists are able to recognize circulating non-hematologic cells (CNHC) without malignant characteristics and identify CTC according to cytopathologic criteria. (El-Heliebi et al., 2013; Hofman et al., 2011)

Based on the ISET filtration enrichment of CTC, blood samples were filtered through 8µm pores (isolated by size – ISET technology) and CNHC were evaluated separately according to their states. Clusters (cellular aggregates) or single cells and were divided into 3 classes, malignant features (CNHC-MF), uncertain malignant features (CNHC-UMF) and benign features (CNHC-BF) according to cytopathologic criteria fulfilment.

Single CNHC has been usually considered as malignant as per following criteria:

1. Nuclei larger than 24µm (3 times higher than 8 µm pore size) (Kamal et al., 2019; Paterlini-Bréchot, 2014; El-Heliebi et al., 2013; Hofman et al., 2011
2. Cell size at least 1.5 times larger than white blood cells (Kang et al., 2018; Nam et al., 2016; Paterlini-Bréchot, 2014)
3. Irregular nuclei and nuclear membrane (Kamal et al., 2019; Kang et al., 2018; Nam et al., 2016; El-Heliebi et al., 2013; Hofman et al., 2011)
4. Anisonucleosis (ratio >0.5) (Kamal et al., 2019; El-Heliebi et al., 2013; Hofman et al., 2011)

5. High nuclear/cytoplasmic ratio (Kamal et al., 2019; Kang et al., 2018; Nam et al., 2016; El-Heliebi et al., 2013; Hofman et al., 2011)
6. Presence of 3-dimensional sheets (Kamal et al., 2019; El-Heliebi et al., 2013; Hofman et al., 2011

Although these criteria are usually taking into account, all of them are rarely present in the malignant cells. Cells with malignant features are characterized by presence of certain amount of these features. (Kamal et al., 2019; El-Heliebi et al., 2013; Hofman et al., 2011)

Incorporation of morphological CTC testing to routine cytological laboratories has seems to be the next step in this area. This field includes identification based on established criteria of cells structure in combination with labelling markers. These institutions perform these activities on cytology specimen therefore intergration of CTC identification into clinical practice can be easily applied. (Lowe, 2018)

Studies have suggested cytomorphologic examination is relevant in the field of CTC detection. Although it may improve CTC presence, other molecular or immunological methods should be added into evaluation process to confirm existence and features of CTC. (El-Heliebi et al., 2013; Pantel et al., 2012; Hofman et al., 2011)

Using nucleic acids for CTC characterization has become popular in last decades. (Table 8) Several innovative methods became a standard diagnostic tool nowadays, e.g. mutations, rearrangements or amplification by PCR, fluorescence *in situ* hybridization (FISH), sequencing, massive parallel sequencing, comparative genome hybridization (CGH), or gene expression analysis. (Cortés-Hernández et al., 2020; Rossi and Zamarchi, 2019; Watanabe et al., 2018) (Table 8)

To examine RNA, gene expression analyses provided by quantitative real-time polymerase chain reaction (qPCR) belongs to the most commonly used methods. It enables researchers to identify expression of genes (tumor-associated; chemoresistance-associated) in particular cancer types' samples, enabling their identifications. (Table 8) New genes have been found to be expressed as increased or decreased in differently advanced oncological diagnoses; the most researched

are BC, CRC, prostate and lung cancer samples. Changes in the expression degree of tumor-specific or tumor-associated genes may be then monitored throughout whole treatment process. (Krebs et al., 2014; Yu et al., 2013; Chen et al., 2013; Dotan et al., 2009)

To examine DNA, PCR has been also used for detection of known DNA mutations in tumor cell samples. (Table 8) Particular gene mutations are often associated with worse survival, cancer behaviour or therapy resistance which may bring a valuable information for clinicians to support the choice of the most suitable way of treatment. In CRC some patients' specific mutations in KRAS and PIK3CA genes have been detected through specific PCR reactions. (Harouaka et al., 2014; Gasch et al., 2013)

Next generation sequencing (NGS) has brought a completely new insight into molecular characterisation of CTC and tumor disease themselves. It enables researches to demonstrate tumor cells and CTC heterogeneity; it enables disease monitoring in time and stresses the possibility of adapting treatment strategies for an individual patient in a process of personalized and precision medicine. (Table 8) CTC may provide a better way how to understand an actual real-time status of disease. In several research publications some important mutations detected in primary tumor and metastasis tissue were found to be present in CTC, too. Moreover, mutations which were detected in CTC first, have been also confirmed in the primary tumor and metastases of the patient. NGS may be also helpful in the process of identification of resistant cancer types which could significantly increase number of patients responding to administered therapies. (Heitzer et al., 2013; Lianidou et al., 2013; Powell et al., 2012; Gerlinger et al., 2012)

FISH is a cytogenetic method based on nucleic acid complementarity. Labelled probes specifically bind to the particular part of RNA and DNA sequences in heterogeneous population of cells. It is an appropriate technique for detection of several types of mutations (amplifications, deletions, etc.) in cytological samples. (Table 8) It is one of the most known and used approach is examination of HER2 status in BC CTC where different HER2 status in primary tumor and CTC within one patient has been reported. (Zhang et al., 2016; Harouaka et al., 2014)

Table 8: Presentation of laboratory methods used in molecular-genetic assays. (taken and adapted from Soekojo et al., 2018; Tomac et al., 2017; Cote and Datar, 2016; Khodakov et al., 2016; The Bench Staff, 2015; Furrer et al., 2015)

Method	Description	Advantages	Disadvantages
PCR **RT-PCR** **qPCR**	Method based on exponencial amplification of target DNA; relative quantification of target DNA sequences through real-time monitoring of the PCR amplification process	Rapid and quantitative analysis of gene amplification; small quantities of DNA fragments are required; flexible, cost- and time-efficient technique; dynamic range and an accurate quantification; high molecular sensitivity	Not possible to gain information regarding morphology; accurate CTC number can not be estimated; RNA fragmentation; nability to perform highly multiplexed assays
NGS	High-throughput sequencing followed by mapping and counting the sequence of interest to determine absolute or relative copy number	Identification of both large and small gene(s) alterations (point mutations, rearrangements, insertions/deletions); suitable for nucleic acid analysis and diagnostic requiring multiplexed analysis of many genes and their variants	Intrinsic error rate due to signal ambiguity, enzyme infidelity, imperfect deprotection; sequencing errors complicate the calling of variants, especially low frequency ones
(F)ISH	Hybridization of labelled nucleotide sequence to a complementary RNA or DNA target sequence	Can be applied to DNA and RNA; study of specific gene amplification, deletion, CNVs and/or gene rearrangements; ability to vizualize genomic alterations, same sample could be used for repeated imaging for different genes behing; high sensitivity	Time-consuming; expensive equipment for signal detection and recognition; difficult to integrate to routine diagnostic laboratory; requirement of well-trained personnel; inratumor heterogeneity can be missed; observers discrepancies
CGH	Comparision of specimen of interest and reference samples – ability to provide	Large aberations detection; ability to assess genome-wide copy number changes;	Expensive, requirement of educated personnel, not standard methodology; unability

	genome-wide information of copy number variations along each chromosome	whole genome analysis; high resolution	

PCR, polymerase chain reaction; RT-PCR,reverse transcriptase polymerase chain reaction ; qPCR, quantitative real-time polymerase chain reaction; CNVs, copy number variations; NGS, next-generation sequencing; (F)ISH, (fluorescence) *in situ* hybridization; CGH, comparative genome hybridization

This availability of advanced technologies enables to analyse genomes of individual CTC derived from clinical samples. Tumor heterogeneity assess is clinically important for proper management of cancer patient and novel and efficient treatment concept development. (Schaffner et al., 2020; Cote and Datar, 2016)

1.8.2.2 Immunohistochemistry

Immunostaining is one of the most widely used techniques mainly because of its easy integration within standard immunohistochemistry in pathology departments. Similarly, immunostaing used by CellSearch® system enables appling simple criteria for CTC identification, e.g. positive expression of cytokeratins, negative expression of CD45 and positive nucleus staining by fluorescent DAPI dye qualifies captured cells to be evaluated as a CTC. (Cristofanilli et al., 2004)

Clinically important markers may be also examined by immunostaining technology. Their prospective expression is crucial in therapy decision. This method can be used in HER2 and EGFR expression status of CTC evaluation, in BC and prostate cancer, respectively. Also, expression of keratins (e.g. KRT19) in CTC has been connected to worse patient outcomes. (Harouaka et al., 2014; Lazar et al., 2012)

Stem cells markers status has been also examined in enriched CTC fractions by immunostaining/immunohistochemistry. Proteins known as associated with stem cell characteristics such as CD44+/CD24-, CD133+ or aldehyde dehydrogenase (ALDH)+ have been present in CTC, during immunostaining procedure. Except of stem cells markers, expression of mesenchymal markers such as TWIST, vimentin or N-cadherin has been investigated due to their role in EMT and metastatic process. (Poruk et al., 2017; Bacelli et al., 2013; Hou et al., 2013; Armstrong et al., 2011; Kallergi et al., 2011; Lecharpentier et al., 2011)

1.8.2.3 Functional assays

Invasion capacity of CTC is one of the most important information on tumor cell behaviour. These cells can be studied from functional point of view mainly due to considerable progress in both *in vitro* and *in vivo* CTC expansion. Several technologies have been developed for functional CTC testing. (Alix-Panabières et al., 2016; Pantel and Alix-Panabières, 2016)

The principle of functional assays is enrichment of viable CTC and ability of their following cultivation. Our group is using MetaCell® device for size-based enrichment of CTC. This technology is enabling to enrich CTC without any modifications on polycarbonate membrane. After enrichment, these cells are cultivated on the membrane for long-time. This approach allows other analysis to be performed. (Klezl et al., 2020; Kiss et al., 2018; Kolostova et al., 2017; Jakabova et al., 2017)

EPISPOT (EPithelial Immuni SPOT) assay have been developed to distinguish between apoptotic and viable CTC isolated from peripheral blood of cancer patients. In the EPISPOT assay cells undergo short-time cultivation on a membrane coated with antibodies that capture secreted/released/shed proteins and those are then detected by secondary antibody labelled with fluorochromes. This assay has been already used for different tumor types including BC, prostate, ovarian, colon cancer or melanomas. (Alix-Panabieres, 2018; Zhang et al., 2016; Alix-Panabières and Pantel, 2015; Ramirez et al., 2014) Published results regarding EPISPOT assay concluded this technique allows establish permantnent cells lines from CTC of cancer patients. Kuske et al. studies CTC of high-risk prostate cancer patients. CTC detection by EPISPOT before radical prostatectomy correlated with PSA serum values and clinical tumor stage. (Kuske et al., 2016) Group of Cayrefourcq et al. studied circulating melanoma cells (CMCs) in patients with metastatic melanoma. CMCs were detected by EPISPOT assay based on their secretion of S100 protein (S100-EPISPOT) and compared with CellSearch®. Although more CMCs-positive patients were detected in S100-EPISPOT assay, results from CellSearch® showed association with overal survival (OS). (Cayrefourcq et al., 2019)

Fluoro-EPISPOT has been optimized for single CTC and its name was changed to EPIDROP. This technology is expected to be more reliable and sensitive in comparison with EPISPOT and can significantly contribute to personalized "oncogram" that might help to improve clinical management of patients with tumor disease. (Cayrefourcq et al., 2019; Alix-Panabieres, 2018)

CTC-derived xenograpfts or CTC derived cell lines are methods to explore vivacious characteristics of CTC obtained from cancer patients at relevant time during disease development. These assays types can clarify functional features of CTC along with *in vivo* and *in vitro* pharmacological testing. Lab of Girotti et al. studied tumorigenic potential of melanoma CTC by injecting enriched CTC from 47 patients' blood samples into mice with success rate of 13% (6/47). It was also reported CTC retained metastatic, histopathologic and immunohistochemical features after injection to mice. The assay can be consider as new tool to study of melanoma biology in patients with advanced stages. This group could also suggest potential way of overcoming the cases when tumors are inaccessible. (Girotti et al., 2016) Group of Pereira-Veiga developed CTC-derived xenograft from advanced TNBC patient. This study demonstrated tumorigenic potential of TNBC CTC and suggested CTC-derived xenografts as attractive *in vivo* model to obtain a detail understanding of tumor biology. (Pereira-Veiga et al., 2018) Recent study of Vishnoi et al. published establishment of TNBC model with liver metastasis by CTC-derived xenograft method. This model recapitulated CTC biology for four sequential generations of mice and helped to reveal 597 genes specific to the TNBC liver metastasis. One of the outcomes was prediction of 6 biomarkers with clinically available drugs. This approach has a great potential in improvement of TNBC patients' outcome. (Vishnoi et al., 2019)

1.8.3 Prognostic value of circulating tumor cells

CTC have been considered as prognostic biomarker in various cancer types. Prognostic significance has been established using immunohistochemistry and qPCR detection methods. Prognostic value of CTC load was studied in patients with metastatic carcinomas by CellSearch® technique as first.

A breakthrough study was organized by Cristofanilli et al. in 2004 (Cristofanilli et al., 2004), 177 patients with mBC were tested before a new line of treatment and at the first follow-up visit. 7.5ml of blood was taken and threshold was established to 5 cells. This research work confirmed an independent character of CTC as a biomarker in progression-free survival (PFS) and OS in patients with this diagnosis. The same approach was also applied for other diagnosis.

In a hormone-refractory prostate cancer study, 64 patients were analysed. It was confirmed that 5 and more CTC in 7.5ml of blood were independent predictors of OS. (Okegawa et al., 2009) In another study, CTC were enumerated in 430 patients with metastatic CRC by the same approach before and during therapy. Threshold for CTC level was estimated to 3 CTC/7.5ml of peripheral blood. It was found CTC count before and during treatment was an independent predictor of PFS and OS in patients with this diagnosis. (Cohen et al., 2008)

In the case of BC metastatic diseases, many studies have been performed for CTC prognostic value confirmation by CellSearch® approach, similarly for metastatic diagnosis of lung, bladder, pancreas, ovarian, head and neck or hepatocellular cancer. (Andree et al., 2016; Balic et al., 2013; Krebs et al., 2011; Miller et al., 2010; de Bono et al., 2008) CellSearch® system was also used for CTC counting in non-metastatic diagnosis including BC, CRC, oesophagus or bladder cancer, it has been found that amount of CTC was much lower. To overcome the obstacle of the low CTC number in non-metastatic patients, bigger volumes of blood for CTC examination could be considered. (Andree et al., 2016)

Other CTC detection methods have also suggested prognostic significance of CTC monitoring among oncologic patients. PCR-based methods without immune-based pre-enrichment step have found to be helpful and sensitive tool in their identification. For peripheral blood mononuclear cells fraction and enriched CTC fraction, qPCR analyses have been used to confirm CTC presence as well. qPCR data were compared in the patients' groups of both early and metastatic cancer diagnosis. (Skerenova et al., 2017; Tewes et al., 2015; Andreopoulou et al., 2012)

One of the genes tested by qPCR quite exetensively is KRT19. KRT19 mRNA analysis was provided by Stathopoulou et al., in 77 patients with early BC after

surgery and 47 previously untreated patients with mBC who were tested before and after chemotherapy. Study data confirmed CTC as promising marker reflecting response to adjuvant chemotherapy. (Stathopoulou et al., 2003) Studies with similar approach have been performed where additionally gene expression of other markers have been examined such as HER2, MGB in patients with early stage BC. Poor clinical outcome and reduced disease-free survival (DFS) and OS have been associated with higher level of KRT19 alone or in combination with other markers. (Xenidis et al., 2009; Ignatiadis et al., 2007) Immunomagnetic separation methods followed by qPCR have confirmed that patients with CTC are displaying poor prognosis. (Molloy et al., 2011; Reinholz et al., 2011)

Other markers have been used for CTC characterisation using qPCR before, during and after follow-up period in studies of CRC patients. The studies confirmed prognostic significance and clinical utility in response monitoring. (Balic et al., 2013; Matsusaka et al., 2011; Iinuma et al., 2011; Rahbari et al., 2010; Papavasiliou et al., 2010) Studies of metastatic CRC have revealed CTC presence is associated with decreased OS in patients with liver metastasis.

Recent studies have also support idea of CTC as prognostic marker. Cristofanilli et al. performed retrospective analysis of 2436 patients with mBC. Patients with ≥ 5 CTC and < 5 CTC were divided into „aggressive“ and „indolent“ group based on CTC results, respectively. Patients in indolent group had longer OS (36.3 months vs. 16 months) regardless of disease subtype than patients in aggressive group. (Cristofanilli et al., 2019) In addition to CTC count, prognostic significance was demonstrated also in molecular field. Strati et al. studied prognostic significance of EMT-associated and stem-cell markers in EpCAM+ CTC. Significantly lower DFS and OS was found in patients with HR- primary tumor and those with more than 3 positive lymph nodes that overexpressed TWIST in EpCAM+ cells. The group also revealed overexpression of TWIST1 and stem-cells (CD24, CD44, aldehyde dehydrogenase 1 (ALDH1)) transcripts present prognostic information in early BC patients. (Strati et al., 2019)

Various methods used for CTC prognostic value establishment associated higher number of CTC with worse prognosis of both disease development and patients'

survival. (Fabisiewicz et al., 2020; Hugenschmidt et al., 2020; Xun et al., 2020; Qiao et al., 2016; Andree et al., 2016)

1.8.4 Predictive value of circulating tumor cells

CTC detection has become tool to monitor real-time state of disease without the need of an invasive biopsy. Deeper investigation of CTC enumeration has revealed their presence may provide more information about treatment response in comparison with standard radiologic techniques, including computed tomography (CT) or positron emission tomography (PET). CTC have found to allow earlier non-responsiveness detection during treatment course and avoid interreader variability for evaluation of radiographic scans, so they have been considered to become helpful in monitoring and treatment. (Alama et al., 2014; Usiakova et al., 2014; Balic et al., 2013; Budd et al., 2006) This predictive potential approach has been drawing attention. Early relapse detection might provide to spare patients the adverse effects of ineffective therapy and also treatment costs may be decreased by way of earlier ending of failed therapy. (Balic et al., 2013; Budd et al., 2006)

1.8.4.1 Circulating tumor cells enumeration

Patients with various BC diagnosis types have been tested for a potentially predictive role of CTC as a part of their therapy monitoring. Nolé et al. used the CellSearch® system for long-term detection of CTC in 80 mBC patients. The study hypothesis suggested clinical response may be indicated by changes in CTC enumeration and they can work as surrogate parameters during targeted therapies for response evaluation. (Nole et al., 2008) Another study analysed 235 mBC patients with various therapy regimens by CellSearch® approach. Their results showed little survival benefit from first-line endocrinne treatment and small benefit of chemotherapy alone in the cases of women with higher CTC counts. (Guiliano et al., 2011) Recently, Ma et al. evaluated 187 BC patients receiving chemotherapy by flow cytometry. Peripheral blood was withdrawn in several time points (before chemotherapy, a day before the third cycle of chemotherapy and a 1 week after the third cycle of chemotherapy). CTC numbers was examined for short- and long- term indicator of therapy effectiveness. Higher CTC numbers presented worse prognosis

after chemotherapy and shorter overall survival in tested BC cohort of Ma et al. (Ma et al., 2017)

Similarly, a predictive role of CTC in patients with lung cancer has been thoroughly investigated. Hirose et al. investigated CTC by CellSearch® method in 33 metastatic non-small cell lung cancer (NSCLC) patients. This study found much higher rate of tumor progression in patients with CTC than in CTC negative ones. (Hirose et al., 2012) Punnoose et al. have showed predictive significance of CTC during treatment in connection to radiological response and clinical outcome. In this study 41 patients with advanced NSCLC treated with erlotinib and pertuzumab were attended. An informative role of CTC cancer prediction has been suggested by study which noticed dramatic decrease in CTC counts at the second cycle of chemotherapy. (Punnoose et al., 2012) Shen et al., have studied 80 patients with small cell lung cancer (SCLC). This group has shown reduction of CTC numbers after two cycles of chemotherapy as a potential predictor of chemotherapy response. CTC could become indicators of treatment efficiency monitoring in patients with lung cancer. (Shen et al., 2017; Muinelo-Romay et al., 2014; Alama et al., 2014)

1.8.4.2 Molecular characterization of circulating tumor cells

Except of CTC enumeration, their molecular analysis represents a significant contribution to suitable strategy choice towards treatment process. Detection of new therapeutic targets and resistance mechanisms could be usually provided at the DNA, RNA and protein levels.

At the DNA level, important mutations in key genes have been already described. Many various mutations have been already assigned as critical. In the case of cancer patients, change in KRAS gene is probably one of the well-known gene changes, mainly because mutations in this gene affect anti-EGFR therapy blocking the proliferative pathways in general. KRAS gene mutations varies between not only patients but also patient itself. (Alix-Panabieres and Pantel, 2016; Mostert et al., 2013; Casch et al., 2013) BC therapy has often relied on HER2-targeted treatment process so activation of PI3K pathway has been also studied in this context. A strong heterogeneity in PIK3CA mutational status in CTC has been reported for

individual patients. (Ibrahim et al., 2015; Mukohara et al., 2015; Polzer et al., 2014; Pestrin et al., 2014) Studies have also focused not only on tumor DNA heterogeneity between patients but also on intra-tumor variability. Mutational status has been tested also before and after therapy where only few variants were shared in samples. (De Luca et al., 2016) Several very interesting and complex observations have been published last year in a Nature science journal. This paper suggests opportunities of potential for early diagnosis due to mutation timing that may occur decades prior to diagnosis. (The ICGC/TCGA Pan-Cancer Analysis of Whole Genomes Consortium, 2020)

Molecular analysis includes also gene expression examination. It is usually tested on RNA and/or protein levels. Significance of gene expression analysis has been already suggested by various CTC studies. Specific genes have been studied to reveal presence and character of CTC and tumor disease itself. Information obtained from these analyses can be useful mainly in the terms of drug sensitivity and resistance. (Alix-Panabieres and Pantel, 2016)

Studies of prostate cancer have identified patients with truncated form of AR, known as Arv7, whose presence in CTC samples could predict failure of antiandrogen therapy with enzalutamide and abiraterone treatment. (Steinestel et al., 2015; Antonarakis et al., 2014) In the case of lung cancer, aberrant expression of EGFR can be result of numerous mechanisms such as epigenetic modifications or increase in copy number variations. EGFR overexpression has been described in 40-80% of NSCLC patients and is correlated with disease progression, decreased survival, lymph node metastasis and poor chemosensitivity. Patients with high EGFR expression have taken an advantage of clinical benefit and increased overall survival from monoclonal anti-EGFR antibodies, such as cetuximab, receiving. (Hopper-Borge and Wangari-Talbot, 2013; Pirker et al., 2012)

1.8.5 Circulating tumor cells testing during therapy

Monitoring of CTC has been found as a useful tool in various parts of treatment process. Surgery still belongs to the most common treatment procedures of cancer diseases if diagnosed in early stages. This type of procedure is the most ordinary

strategy in majority of early-stage NSCLC patients, the primary treatment of patients with melanomas or the first choice, in most thyroid cancers. Despite it's relatively often uses in tumor diseases, many unwelcome consequences may occur. (DeSantis et al., 2014) In addition to surgery, other therapies have been widely used in various tumor diseases. This treatment usually includes receiving of chemotherapeutics, radiotherapy, immunotherapy, hormone or targeted therapies which can be administered individually or in combination with surgery or each other. (National Cancer Institute, 2017)

Detection of CTC before and after surgery procedure has been usually associated with poor outcome. (Van Dalum et al., 2015) Recent studies have connected chemotherapy and targeted therapies with higher impact on CTC numbers in comparison to surgical treatments. Reduction of CTC-positive rates could be likely after neoadjuvant, adjuvant, palliative or combination treatment but not after surgery regardless of CTC detection method used. Long-term studies suggested shorter recurrence-free survival (RFS) and OS of patients with CTC presence in pre-surgery, one and two years after surgery but not in 1 week after surgery samples. Based on these results, it has been concluded chemotherapy treatment should be highly recommended for patients with initially CTC positive status after surgery to increase a chance of more favourable outcome. (Yan et al., 2017; Van Dalum et al., 2015)

Numbers of studies monitoring therapy efficiency using CTC have been performed in BC. Study of Coumans et al., have concluded that the aim of cancer treatment should be the elimination of all CTC; while reduction to zero CTC can be seen after 4 to 6 weeks. To reach this aim, 10 to 12 weeks of therapy may be needed for some patients; and if the number does not decrease within this time span, treatment is not effective. (Coumans et al., 2012) CTC status has been monitored also after start of treatment. It has been found out in the persistently increased CTC after 21 days of first-line chemotherapy; more effective treatment should be highly recommended. Research has suggested CTC which are determined shortly before the second cycle of chemotherapy have been found as early predictor of treatment outcome. (Smerage et al., 2014; Martin et al., 2013; Rack et al., 2012)

Molecular testing of CTC in regular intervals can also reveal important information. (Figure 14) Correct timing may be useful in mainly in therapy efficiency monitoring. Current studies have been discussing new markers to distinguish patients between responders and non-responders. This information is crucial mainly in the term of disease cure, cancer slowdown and cancer progression. Numbers of markers have to be known for their contribution to disease progression. (Bobek and Kolostova, 2018; Leong et al., 2015)

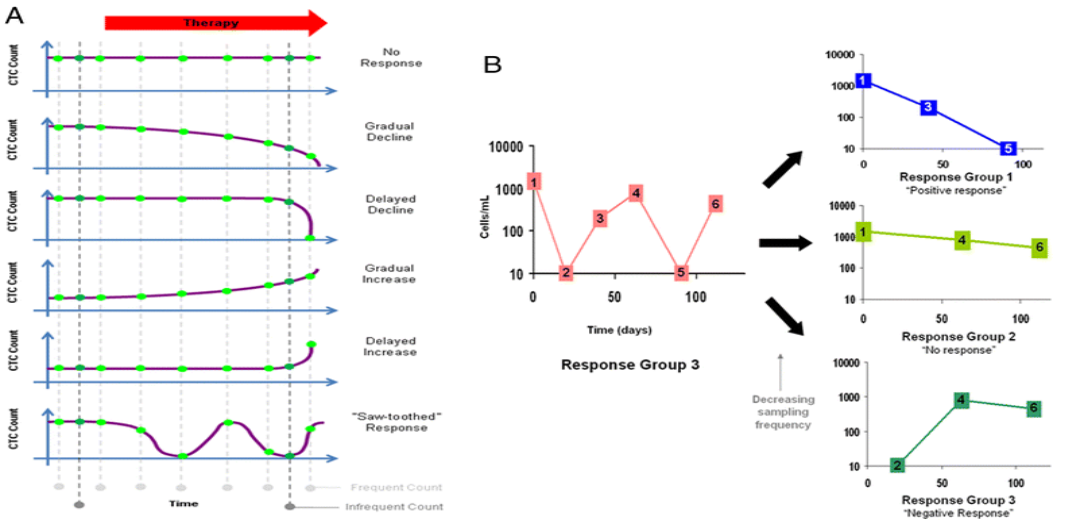

Figure 14: Schematic presentation of circulating tumor cells (CTC) response patterns in cancer patients during therapy courses. A) Hypothetical CTC count options in cancer patients during therapy. Light green cycles represent frequent sampling times, dark green cycles represent infrequent sampling times. B) Representation of potencial responses interpretations, simulation of sampling frequency when is halved from 6 to 3 sampling points. (adapted from Leong et al., 2015)

There is no right or wrong frequency of CTC sampling. It is necessary to keep in mind the consequences of inappropriate timing of CTC testing. (Figure 14) Although low frequency of CTC testing can result from wrong assumption or delayed diagnosis, opposite situations have usually no more advantages. Too frequent withdrawals and CTC examination can lead to CTC oscillation because of their usually endless changing dynamics which can cause misleading trend. (Figure 14) Numbers of studies should be done for the most appropriate CTC analysis frequency determination by the highest possible withdrawals number to prevent loss of essential information. (Leong et al., 2015)

1.8.6 Circulating tumor cells in breast cancer

For better understanding of metastatic process and spread of disease, biological features of CTC should be revealed as accurate as possible. Since detection of CTC has been usually correlated with tumor burden, it has been believed they are more frequent in the cases of mBC. (Bidard et al., 2016) Studies have also suggested CTC release starts early during progression of BC. These cells are shed from primary tumor and are distributed to other organs which results in metastatic lesion formation. (Gkountela et al., 2016; Hüsemann et al., 2008)

Breast tumor characterization according to HR status and HER2 status has become a standard part of BC evaluation. Based on these results, patients can receive targeted therapy. Although these examinations of primary tumor can be helpful in therapy selection, CTC and primary tumor consistency are rare. CTC are also unusually very heterogeneous not just between patients but also within the patients' themselves. This diversity is manifested in various characteristics such as higher tumor-associated markers expression or difference in tumor-seeding potential. Variability between both CTC and primary tumor and CTC themselves can results from very dangerous consequences for patients. One of the most urgent is their unresponsiveness to therapy. (Paoletti et al., 2018; Polasik et al., 2017; Reinhardt et al., 2017; Gkountela et al., 2016)

Researchers who study these issues in BC are often focused on ordinarily tested HR characteristics. The reason for this approach is the evidence of endocrine therapy effectivness as of a leading treatment choice in HR-positive BC of all stages. One of the examples is the analysis of ESR expression in BC patients with ESR-positive BC diagnosis. Most of the studies have revealed lack of ESR expression in CTC which may be a mechanism explaining their resistance to endocrine therapy such as tamoxifen or raloxifene. (Kabel, 2017; Reinhardt et al., 2017; Paoletti et al., 2016; Babayan et al., 2013) Then as well, HER2 expression between primary tumor and CTC has been studied and compared. The results revealed inconsistency between tissue and liquid biopsies.

Unpredictable behaviour of HER2-positive and HER2-negative CTC has been proved by number of studies. Jordan et al., has confirmed HER2-positive/HER2-

negative spontaneous interconversion in BC CTC but the work has proved this CTC feature at the level of a single CTC. Surprisingly, single HER2-negative CTC firstly proliferated slowly but after acquired HER2-positive daughter cells rapid interconversion thereafter. Similarly, single HER2-positive CTC with rapid proliferation generated HER2-negative CTC progeny which rose slowlier. (Jordan et al., 2016) Examination of HER2 status is important mainly because of its linkage to more aggressive disease behaviour and significantly decreased survival rate of patients. Paper of Jordan et al. also highlighted the importance of CTC molecular testing in real-time. Targeted therapeutics such as trastuzumab or lapatinib can improve patients' outcome. (Kabel, 2017; Gkountela et al., 2016; Jordan et al., 2016; Gutierrez and Schiff, 2011; Pestrin et al., 2009)

Other phenotypic characteristics to be significant in CTC include mesenchymal features, stem-cell like features or clusters presence in peripheral blood. Although CTC that undergo EMT usually represents a small fraction of cells which disseminated from tumor tissue, this step is critical for metastatic cascade. CTC with EMT markers have been detected in BC patients often during disease progression. In most of these studies, detection of EMT markers shorter PFS and their presence have been usually connected with patients who do not respond to therapy. (Krawczyk; et al., 2014; Mego et al., 2012; Yu et al., 2011; Aktas et al., 2009) Surprisingly, CTC monitoring during treatment and disease progression has revealed reversible shifts between these two features which may suggest EMT occurrence as result of therapy failure. Other explanation has suggested interesting individual cell diversity in mesenchymal and epithelial markers. It has been confirmed co-expression of both types of markers in the same cell in BC patients. (Gkountela et al., 2016; Yu et al., 2013; Kallergi et al., 2011; Armstrong et al., 2011) Reversibility of EMT process has been suggested by detecting CTC in BC patient who was positive for vimentin (VIM) but this marker was not detected in metastasis. (Krawczyk; et al., 2014; Armstrong et al., 2011)

Presence of cells with stem-cell like properties has been proven in patients with BC. They have been associated with aggressive behaviour of tumor. Confirmation of their responsibility in the process of tumor cells dissemination and metastasis has been presented by researchers. (Giordano et al., 2012; Barrière et al., 2012; Mego

et al., 2012; Raimondi et al., 2011) CTC with higher expression of not only stem cell-like but also EMT markers have been found in peripheral blood of patients with both early and mBC. Studies have indicated population of CTC with stem-cell characteristics is produced by EMT. (Mego et al., 2012; Giordano et al., 2012; Morel et al., 2008) According to multiple studies, CTC with proven expression of stem cells markers have been considered as one of the possible explanations for therapy resistance and failure in BC. (Krawczyk; et al., 2014; Monteiro and Fodde, 2010)

CTC clusters belong to other typical characteristics of CTC in patients with BC. There are two or more CTC which are bound together and may be displaying from 23- to 50- fold higher potential of metastasis forming than a single CTC. (Gkountela et al., 2016; Aceto et al., 2014) Presence of CTC clusters in peripheral blood of patients has been clearly associated with shorter PFS, metastatic-free survival (MFS) and OS in various types of BC compared with individual CTC. (Mu et al., 2015; Paoletti et al., 2015; Aceto et al., 2014) Possible hypothesis about their origin has been tested and results from surprising suggestions. These cells have been rather derived from tumor tissue or raised from oligoclonal expansions of cancer cells assembles than aggregation of single CTC or individual CTC proliferation. (Gkountela et al., 2016; Lu et al., 2015) Further analysis of single and clustered CTC has revealed difference in expression of protein called plakoglobin which has been responsible for holding cancer cells together and also has been significantly increased in CTC clusters. (Figure 15) Indeed, analysis of single and clustered CTC from the same BC patient showed negative and positive result for plakoglobin, respectively. (Aceto et al., 2014) Elevated expression of this biological macromolecule in primary tumors of BC patients was also associated with decreased MFS which leads researchers to conclude heterogeneity of plakoglobin expression in primary tumors results in different types of CTC production. (Gkountela et al., 2016; Lu et al., 2015)

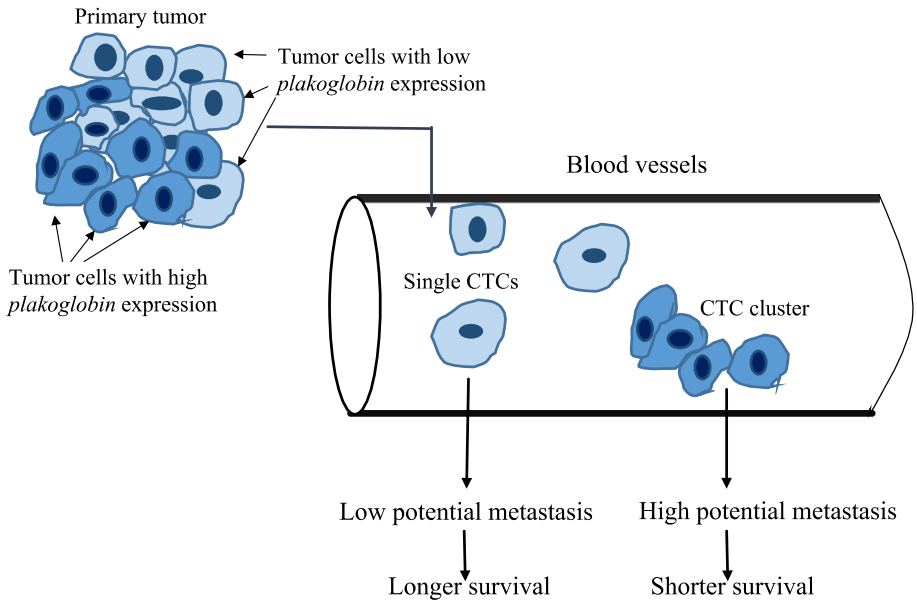

Figure 15: Presumed model of plakoglobin location, CTC types, metastatic capabilities. Cancer cells that extensively express plakoglobin escape from tumor tissues, enter and circulate in clusters in the bloodstream. These clusters promote metastasis to other sites which can lead to worse patients survival. CTC, circulating tumor cell; CTCs, circulating tumor cells. (adapted from Lu et al., 2015)

Plakoglobin has been considered also as oncogenic or tumor-suppresive molecule but this hypothesis has never been confirmed. Although plakoglobin expression was negatively associated with prognosis in the mean of BC progression, increased expression of this protein enables CTC to circulate in clusters which raise a chance of distant metastasis formation and often results from worse survival. (Figure 15) (Lu et al., 2015) On the other hand, other research study has also revealed cytokeratine 14 (KRT14) as a key regulator of metastasis through CTC clusters. They have found cells positive and negative for KRT14 were usually enriched during metastasis phases associated with systemic spread and proliferation in BC patients, respectively. (Cheung et al., 2016) Results of these studies have indicated analysis of CTC clusters has an independent prognostic value and CTC are remarkably effective precursors of metastasis in BC. Information from this analysis may be obtained at the same time as CTC examination without any extra additional tools or experiments. Although liquid biopsies in BC have been promising for patients' individualized treatment it has been found that clinical value of CTC varies between early and mBC. (Banys-Paluchowski et al., 2016; Gkountela et al., 2016; Mu et al., 2015)

1.8.6.1 CTC in early BC

Most patients with early BC (eBC) have usually good prognosis and survival has been significantly improved nowadays. For continuation of this trend, novel markers have been discovered and investigated to help with treatment process determination. Methods which are used are often based on clinical, pathological and molecular genetics features of tumor and patient. Despite of great improvement, significant cases of problems, such as relapses have been documented in patients with this diagnosis thus new tools for long-term and regular disease monitoring are essential. One of the promising biomarkers is CTC which can add detailed information about patients' real-time disease behaviour and increase chance for cure. Although plenty of studies have been performed to research their potential benefit, some of the CTC eBC-related issues have still remained inconsistent. (Ramos-Medina et al., 2016; Banys-Paluchowski et al., 2016)

Studies regarding the CTC in eBC have mostly reported CTC positivity from 7.9 to 55% of these patients. (Thery et al., 2019; Bidard et al., 2018; Pierga et al., 2017; Hall et al., 2015; Banys et al, 2012; Georgoulias et al., 2012; Molloy et al.,2011; Sandri et al., 2010; Krishnamurthy et al., 2010; Ferro et al., 2010; Riethdorf et al., 2010; Apostolaki et al., 2009) Their presence has been usually connected with decreased of progression-free and overall survival regardless of detection method or blood sampling timing. (Maltoni et al., 2016; Schindlbeck et al., 2013; Lucci et al., 2012; Zhang et al., 2012; Graves and Czerniecki, 2011; Bidard et al., 2010) Researchers have also tried to find a relationship between other clinic-pathological characteristics of disease and CTC but the results are quite inconsistent in the moment. A significant connection between CTC presence and both lymph node and receptors status has brought attention for detailed investigation. On the one hand it has been found that CTC presence was independent of either lymph node status or receptor status. (Krishnamurthy et al., 2010) On the other hand positive correlation between lymph node status and CTC positivity was reported. This was an evidence for prognostic relevancy of CTC presence in the HR positive eBC tumors' patients but not in the ones with HER2-positive and TNBC types. (Rack et al., 2014; Rack et al., 2010) Association between CTC prognostic character and both TNBC and

HER2-positive patients was described, but not confirmed for CTC status and luminal BC tumors. (Hwang et al., 2012) Other papers have also suggested HER2 status of primary tumor can be a trustworthy predictor of CTC presence but this has not been approved by other researchers. (Lang et al., 2009) Several studies have also confirmed CTC as strong independent prognostic factor of lymph node, receptor status and tumor grade and size. (Pierga et al., 2015; Mego et al., 2015; Graves and Czerniecki, 2011; Krishnamurthy et al., 2010)

Discrepancy between features of primary tumor and CTC may seriously affect patients' survival. Several studies have been performed to clarify different HER2 status of CTC and primary tumor in eBC patients. Elevated expression of KRT19 or MGB in CTC has been associated with poorer prognosis. (Graves and Czerniecki, 2011; Ferro et al., 2010) Other studies focusing on this prognostic relationship have suggested certain time limitation - during the first 36 and 48 months CTC positivity may act as a survival predictor, more studies should be performed to clarify this issue. (Banys-Paluchowski et al., 2016; Bidard et al., 2013) Innovatively, CTC enabled to monitor minimal residual disease in patients with early stages of BC and without obvious metastasis evidence. (Maltoni et al., 2016)

Despite an initial examination of tumor characteristics may be usually complex, development and evolution of eBC in the meaning of long-term prognosis may not be estimated precisely. Patients with the same primary tumor features have rarely the same or similar diseases' progress. Promising potential of CTC is to get as much information as possible through CTC profiles at all steps of disease progression to decide as accurate targeting strategy as possible during treatment process. (Ramos-Medina et al., 2016; Maltoni et al., 2016)

Regarding significant number of eBC patients suffering from treatment resistance or relapses, new challenge for CTC in future during treatment monitoring is pending. Most of the studies have agreed on that CTC persistence has meaningful impact on eBC patients' survival. They have been usually connected with shorter DFS, PFS and OS. Although results suggest CTC detection is associated with worse clinical outcome, key time point of blood withdrawal during treatment process is still not clear. (Banys-Paluchowski et al., 2016; Ramos-Medina et al., 2016; Hall et

al., 2015; Rack et al., 2014; Graves and Czerniecki, 2011) Results of studies which have performed real-time CTC monitoring during therapy differ significantly. Associations between pre- and post- treatment CTC results with DFS, PFS and OS have been investigated. A part of studies suggested both pre- and post- treatment CTC analysis is associated with DFS and OS. (Pachmann et al., 2011; Xenidis et al., 2009) Opposite, a part of the publications reported inconsistent results in pre- or post- treatment withdrawals in relationship with patients' outcome. Both shorter DFS and OS were associated with pre-therapy CTC detection while post-treatment CTC observation was connected with reduced DFS. (Rack et al., 2014; Pierga et al., 2008) Different studies have claimed that single one of the pre- or post-therapy sampling is a better predictor of DFS and OS. (Bidard et al., 2014; Rack et al., 2014; Bidard et al., 2010) Additionally, Xenidis et al. have used CTC analysis to evaluate survival benefit between taxane-based and taxane-free group. More favourable DFS was observed in taxane-based patients. This survival advantage was also showed by shifting from CTC-positive to CTC-negative status of significant fraction of taxane-based patients' group. (Banys-Paluchowski et al., 2016; Xenidis et al., 2013)

Achieving of pCR after neoadjuvant chemotherapy (NACT) is usually associated with favourable clinical outcome although it is not an evidence of a complete cure. (Ramos-Medina et al., 2016; Symmans et al., 2007) Both pCR and CTC detection have been investigated for their mutual relationship. Although one part of the studies reported pCR and CTC-negative status for patients with an increased DFS, other researchers reported no correlation between CTC detection and treatment response. (Ramos-Medina et al., 2016; Pierga et al., 2015; Riethdorf et al., 2010)

Discrepancies between CTC presence and pCR have seemed to originate in primary tumor properties which correspond to neither CTC nor possible metastasis characteristics. Although in eBC patient treatment choice is usually selected based on primary tumor features an incredible diversity between primary tumor and CTC is reported nowadays. (Maltoni et al., 2016; Banys-Paluchowski et al., 2016; Ramos-Medina et al., 2016; Graves and Czerniecki, 2011)

Changes in ESR or HER2 status can contribute to endocrine or anti-HER2 therapy resistance, respectively. Particularly, HER2 status has been taken place to detailed

studies. Although numerous publications have showed HER2-positive CTC status in HER2-negative primary tumor which is not rare event in eBC patients, these people are not eligible to treatment targeted against HER2 resulting into higher relapse risk. (Banys-Paluchowski et al., 2016; Riethdorf et al., 2010; Fehm et al., 2009) Studies also associated anti-HER2 therapy receiving with longer DFS and smaller danger of relapses. Anti-HER2 therapy was demonstrated to eliminate HER2-positive CTC regardless of the eBC tumor type. (Georgoulias et al., 2012; Rack et al., 2012) This fact has been currently evaluated in clinical study TREAT CTC Trial (NCT0158677). Patients with HER2-negative primary BC who had detectable CTC after completing both (neo) adjuvant chemotherapy and surgery are eligible. They were randomized in 1:1 ratio to trastuzumab or observation group and treatment response was assessed after 18 weeks of treatment/observation. (ClinicalTrials.gov) Trastuzumab did not decrease the detection rate of CTC in HER2 non-amplified, nonmetastatic BC (Ignatiadis et al., 2018)

Treatment process of eBC requires real-time information on actual disease status. It is necessary to keep in mind potential danger, such as discordance between primary tumors and both CTC and possible metastasis may occure. CTC may be a useful tool to help with real-time monitoring and possibly minimalize consequences of unpredictable cancer behaviour. Moreover, with growing suggestions of early metastasis theory in eBC it is necessary to start disease monitoring from as early point as possible. CTC analysis may allow clinicians not only to recognize patients' disease progression but also to identify those with higher risk of recurrence. Additionally, molecular and genetic characterization of these cells allows evaluation of chemoresistance profiles and therapeutics targets which can enable more efficient and individualized curative regimens. (Maltoni et al., 2016; Banys-Paluchowski et al., 2016; Ramos-Medina et al., 2016; Bidard et al., 2014; Georgoulias et al., 2012; Patil et al., 2010; Carmichael et al., 2010; Benavides et al., 2009)

1.8.6.2 Circulating tumor cells in metastatic breast cancer

CTC studies related to mBC have been provided more often than in eBC. The main purposes of mBC research are to improve patients' life quality and prolong their survival expectance. For fulfilling these ambitions, it is necessary to develop approaches which would be able to abort or at least to slow down tumor progression and reduce side effects of receiving treatments. Despite the confirmed fact of not only phenotypic but also genotypic discrepancies between primary tumor and metastasis occur, most of the treatment choices have been still performed based on primary tumor characteristics. (Banys-Paluchowski et al., 2016; Banys et al., 2012)

CTC are usually detected in the 40-80% of patients with mBC and belong to relatively new tools for monitoring metastatic cancer development. Initial CTC analysis performed before beginning of the palliative therapy demonstrated that CTC presence was a prognosticator of shorter PFS and OS of mBC patients. Subsequently studies have revealed very poor outcome, reduced PFS, OS of mBC patients with persistently elevated CTC number at any point during therapy regimens. (Banys-Paluchowski et al., 2016; Smerage et al., 2014; Graves and Czerniecki, 2011) Moreover, expression analysis of CTC has connected shorter PFS of mBC patients with greater than two multidrug-resistance proteins (MRPs) elevated in comparison with those without. (Gradilone et al., 2011)

Regular tracking of disease evolution belongs to the most important parts of treatment process mainly because of endlessly possible illness changes during therapy process. Moreover, phenotype and genotype of primary tumor, isolated tumor cells in secondary homing sites and metastatic tissue may differ therefore most of the primary tumor biopsies tissues usually do not reflect metastatic sites character. This problem is often seen in the mBC cases regarding hormone receptors and HER2 status which have been intensively studied. (Agelaki et al., 2015; Rack et al., 2012) Determining HER2 status, 31% and 26% discrepancies were found between CTC in comparison with primary tumor and metastasis, respectively. (Wallwiener et al., 2015) Fehm et al., detected HER2-positive CTC in significant number of patients with HER2-negative primary tumors. (Fehm et al., 2010) Moreover, a research focused on anti-HER2 therapy reported promising effects of

this treatment which have been found to significantly contribute to persistent HER2-positive CTC regardless of primary tumor status in mBC patients. (Banys-Paluchowski et al., 2016; Agelaki et al., 2015; Rack et al., 2012)

Studies have also investigated CTC as strong and early predictive marker of chemotherapy response. Not only CTC enumeration but also their genetic profiling seems to become a useful tool to determine real-time status of individual patients' disease progression, treatment efficiency and outcome prediction. Researchers see a big potential of CTC in their contribution to reduce treatment adverse reactions. (Gkountela et al., 2016; Fina et al., 2015; Graves and Czerniecki, 2011) Wide ranges of not just short- but also long-term side effects have been reported during and after receiving of anti-cancer drugs. (Institute for Quality and Efficiency in Health Care, 2016; DeSantis et al., 2014) Early information about ineffective and moreover potentially harmful treatment can significantly contribute to improvement of patients' life quality. This CTC potential can be used to reduce toxic exposure caused by the treatment with little or no effect which can be followed by switching to alternative therapy receiving. (Ramos-Medina et al., 2016)

There are several other biomarkers and imaging techniques used by clinicians that are helpful in disease monitoring and in specifying therapy continuation. In comparison with other markers, CTC have been confirmed as prognostic in mBC regardless of molecular BC subtype, serum markers such as CEA or cancer antigen 15-3 (CA 15-3) have been shown not to improve prognostication in mBC. (Banys-Paluchowski et al., 2016; Bidard et al., 2014) Moreover, interesting experiment has been performed to compare correlation of treatment response by CTC presence and radiologic assessment and also in relationship with OS. Patients without radiologic progression and elevated CTC number had significantly shorter OS than patients with radiological progression and decreased CTC number. Most of the studies have agreed on using CTC as trustworthy alternative biomarker of therapy response. CTC are identifying not only patients with poor prognosis but also those with little or no benefit from actual treatment receiving. (Ramos-Medina et al., 2016; Budd et al., 2006)

1.9 Resistance to anticancer drugs

Even though researchers have made a significant progress in new treatment combinations and mechanisms, therapy failure has been still the consequence of chemotherapy resistance. Resistance strategies have been naturally evolved as protection to prolong survival of living cells by adapting to environmental changes with offensive conditions which is true for chemotherapy, too. The cells are able to achieve this adaptation by either intrinsic or acquired mechanisms. To reach effectiveness, anticancer drugs, also like other medication, need to be absorbed through lipophilic membranes, distributed to cells and tissues through the blood, to target the wanted site. (El-Awady et al., 2017; Blair et al., 2014; Binkhathlan and Lavasanifar, 2013; Holohan et al, 2013; Swanton, 2012; Tiwari et al., 2011)

The most common anticancer multidrug resistance (MDR) mechanisms have been classified into three categories: target-dependent, drug/target-independent and drug-dependent. Factors which are able to influence drug targeting (target translocation, mutation, deletion or amplification) are responsible for target-dependent MDR. On the contrary, drug/target-independent MDR has occurred in the case of drug targeting desensitization by genetical or epigenetical modification of cell signalling pathways. Moreover, MDR dependent on drug has been caused by numerous mechanisms to decrease intracellular drug concentration, including efflux drugs transporters or detoxifying enzymes overexpression which results from decreased uptake or elevated drugs efflux withing cancer cells. (Figure 16) Despite numerous possibilities of MDR which have been already clarified, overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) super-family of transporters has been considered as the most prominent mechanisms among organisms. (El-Awady et al., 2017; Chen et al., 2016; Holmes et al., 2016; Ye et al., 2016; Zhang et al., 2016; Li et al., 2015; Konig et al., 2013; Gillet and Gottesman, 2010)

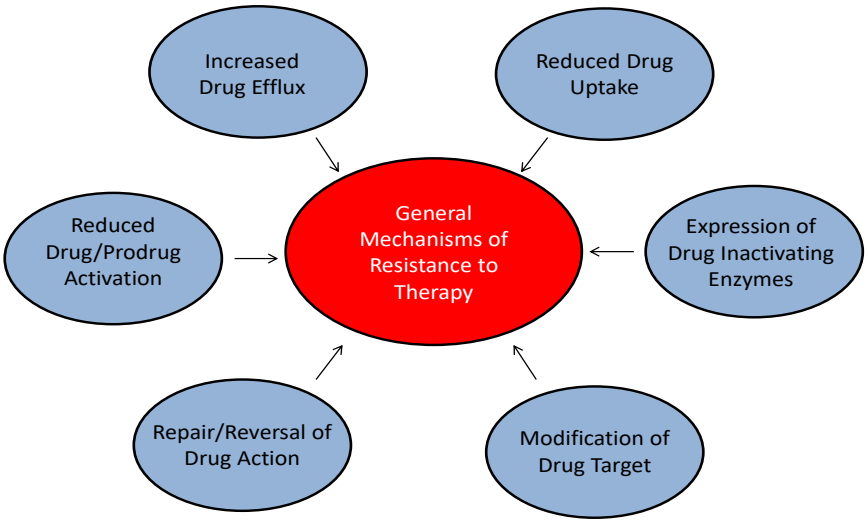

Figure 16: General overview of resistance mechanisms to chemotherapy. (adapted and modified from El-Awady et al., 2017)

Although anticancer treatment includes variety of chemotherapeutics used for specific tumors, cancer resistance mechanisms are usually common. (Li et al., 2016; Akhdar et al., 2012) The ABC transporters are the most abundant superfamily of protein transporters present in all organisms. They are playing an important role not just in efflux but also in drug metabolism. Convetional drug metabolism process is consisting of Phase I (exogenous compounds oxidation/reduction to pro-drugs or inactive actives activation) and Phase II (partially detoxified metabolites conjugation), Phase 0 (entry and exit control) and Phase III (total eradication of the metabolized molecules assurance). All of the phases are responsible for cellular entry and exit of exogenous compounds alteration. (El-Awady et al., 2017)

The ABC transporters present in human are complexes which are categorized based on their sequenced similarities into seven subfamilies, from ABC subfamily A (ABCA) to ABC subfamily G (ABCG) and are consisting of 48 members. Although these transporters are mostly settled on the plasma membrane, their subfamily, ABC subfamily C (MRP/ABCC), are able to provide intracytoplasmic drugs capture and pump them out. The systems are able to decrease various toxins concentration which can cause the chemotherapy failure and cancer progression in the term of tumor disease. ABC transporters subtypes, ABC subfamily B member 1 (ABCB1)

and ABCC subfamily, are the most associated with MDR in various cancer types. (Tang et al., 2016; Chen et al., 2016; Ween et al., 2015; Alakhova and Kabanov, 2014)

Structure of ABC transporters are composed of transmembrane (TMDs) and nucleotide binding domains (NBDs). (Figure 17) Structurally diverse TMDs are hydrophobic and are alternatively able to recognize and translocate different substrates upon conformational changes. NBDs are highly conserved proteins that contain Walker A and B motifs and the signature (C) motif which joins the Walker A and B motifs. These two motifs play crucial role in ATP hydrolysis and energy coupling. (Figure 17) Moreover, ABC transporters can be categorized based on their structure and sequence of the NBDs domain, also known as ABC domains. (Kathawala et al., 2015; Choi and Yu, 2014; Rice et al., 2014; ter Beek et al., 2014; Kunjachan et al., 2013)

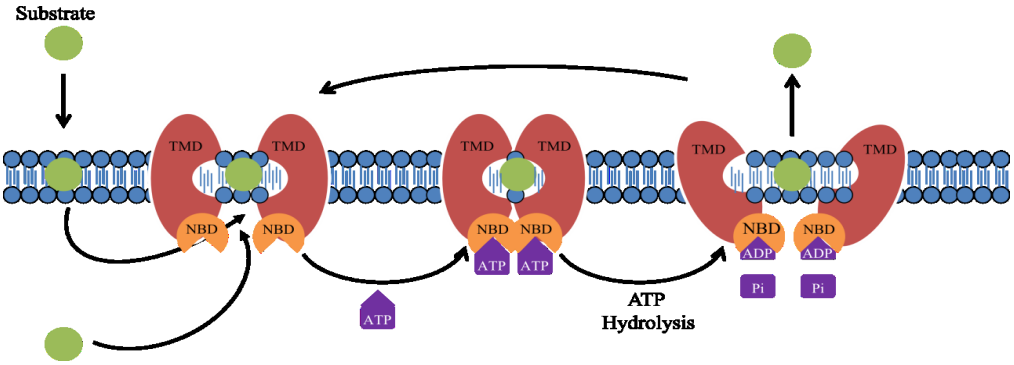

Figure 17: Schematic description of ABC transporters function. TMD, transmembrane domain; NBD, nucleotide binding domain; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, phosphate. (adapted from Chen et al., 2016)

The prominent function of the ABC transporters, efflux, has been summarized into several steps which lead to complex conformational modifications (Weismanova et al., 2012):

1. At the beginning, ABC transporter is in the open form and has high afinity to the substrate. Transmembrane domain (TMD) associates with substrate in both inside the cell or in the cytoplasmatic membrame which results from conformation change and ATP molecule binding.
2. Walker A in the first nucleotide binding domain (NBD) forms a bond with ATP molecule which is stabilized by C motif from the second NBD. This „sandwich“

bond (Walker A – ATP molecule – C motif) facilitates the second ATP molecule binding to Walker A from the second NBD which is stabilized by C motif from the first NBD. These „sandwich“ bonds cause getting the both NDBs closer followed by conformational change of TMDs with substrate from open to closed position. Cavity between TMDs is created. (Figure 17)

3. Energy obtained from ATP hydrolysis is used for rotation of cavity with substrate. Cytoplasmic region is rotating into extracellular environment which is accompanied substrate release into extracellular environment. (Figure 17)
4. Energy obtained from second ATP hydrolysis is used for transporter original returning.

1.9.1 Genes associated with chemoresistance

Despite the fact ABC transporters have performed useful tasks in the cells, they have found to be involved in drug efflux out of the cells. As a consequence, there are more cancer cells with higher expression of multidrug-resistant genes which support intrinsic chemoresistance. (Table 9) Although chemotherapy failure is often a multifactorial process, overexpression of ABC transporters plays very an important role. The activation of ABC transporters has found to be the first and limiting event of the acquired resistance. This event can be a consequence of several mechanisms such as higher amplification of the genes, increased transcription by novel transcription factors, changes in translational efficiency, genes mutations or chromosomal rearrangements. Moreover, treatment options such as radiotherapy or chemotherapy have been found to cause multi-drug resistance efflux tranporters over expression. (El-Awady et al., 2017; Tang et al., 2016; Kathawala et al., 2015; Gazzaniga et al., 2010a) Many studies regardless of cancer types have demonstrated that elevated expression of the ABC transporters in cancer cells causes poor clinical outcome. (Housman et al., 2014; Yanase et al., 2004) Their association with prognosis, prediction, clinicopathological characteristics and tumors behaviour have been extensively studied in patients with various tumor diseases. (Table 9)

1.9.2 Resistance and circulating tumor cells

Transporters contributing to the chemoresistance were also analysed in CTC of various cancer diagnoses. Despite the great progress in tumor treatment options, the major causes of death are still both cancer progression and metastasis. Detailed research of this area is focused on question, if the right cancer cell has been targeted. It has been suggested CTC population is heterogenous and consists of resistant subpopulations which are able to cause distant metastasis. Moreover, the results of resistant markers in CTC have confirmed CTC testing as promising. (Abdallah et al., 2016; Liberko et al., 2013; Gazzaniga et al., 2010b)

Table 9: List of selected genes associated with chemoresistance and their characteristics. Table contains localisation of selected genes in healthy cells/tissues, type of cancer disease that association with selected genes was studied and particular references.

Gene	Occurence	Type of cancer	Reference
MDR1 / ABCB1	liver, placenta, intestine, kidneys, adrenal gland, colon and blood-brain barrier	pancreatic, breast, ovarian, lung, bladder, sarcoma, glioblastoma, colon, leukemia, gastrointestinal tumors, hepatocellular carcinoma, colorectal, liver, osteosarcoma, thyroid tumor, esophageal tumor, testicular germ cell tumors	Kassem et al., 2019; Briz et al., 2019; Cornelison et al., 2017; El-Awady et al., 2017; Sun et al., 2016; Fan et al., 2016; Balaji et al., 2016; Borel et al., 2012; Lu et al., 2012; Nakai et al., 2009; Duan et al., 2008; Lhomme et al., 2008; Lu et al., 2007; Roy et al., 2007; Warmann et al., 2005; Diestra et al., 2003; Sun et al., 2000; Tidefelt et al., 2000; Abolhoda et al., 1999; Del Vecchio et al., 1997; Trock et al., 1997; Mizoguchi et al., 1990
MRP1 / ABCC1	testis, prostate, lung, thyroid, urinary bladder, liver, kidney, regions of the gastrointestinal system, such as pancreas, esophagus and stomach	lung, ovarian, colorectal, esophageal, leukemia, lung, breast, colon, prostate, hepatocellular carcinoma (localized in intracellular membranes of tumor cells-minor)	Briz et al., 2019; Fan et al., 2016; Lu et al., 2015; Zhang et al., 2015; Bagnoli et al., 2013; Yamada et al., 2013; Hlaváč et al., 2013; Zhang et al., 2015; Hlavata et al., 2012; Sodani et al., 2012; Chen and Tiwari, 2011; Roy et al., 2007; Nies et al., 2001; Nooter et al., 1997
MRP2 / ABCC2	hepatocytes membrane, peripheral nerves, gallbladder, CD4+ lymphocytes and in the intestine, apical membrane of epithelial cells of the biliary system and proximal renal tubules	breast, colon, ovarian, lung, hepatocellular carcinoma, renal carcinoma, colorectal, skin melanoma, liver, cholangiocarcinoma, testicular germ cell tumors, leukemia	Briz et al., 2019; Lacy et al., 2015; Zhang et al., 2015; Chen and Tiwari, 2011; Shibayama et al., 2011; Tiwari et al., 2011; Korita et al., 2010; Zimmermann et al., 2008; Gillet et al., 2007; nies et al., 2007; Huisman et al., 2005; Sandusky et al., 2002; Mottino et al., 2001; Rost et al., 2001

MRP3 / ABCC3	highly expressed in the liver, gastrointestinal system, kidney, colon, biliary system, pancrease adrenal glands tract or gallbladder and less in the spleen, tonsils, stomach or lungs	ovarian, hepatocellular carcinoma, colorectal tumor, kidney carcinoma, breast, colon, bladder, lung, testicular tumors, thymomas and gliomas	Briz et al., 2019; Tomonari et al., 2016; Balaji et al., 2016; Lacy et al., 2015; Zhang et al., 2015; Hlavata et al., 2012; Sun et al., 2012; Tiwari et al., 2011; Lagas et al., 2010; Scheffer et al., 2002; Zelcer et al., 2001; Kool et al., 1999; Ortiz et al., 1999
MRP4 / ABCC4	prostate, kidney, urinary bladder, pancreas, thyroid, testis, pancreas and colon cancer, hepatocytes, gastric cells	prostate, renal, gastric, tumors derived from the digestive tract, kidney, pancreatic, ovarian, breast, bladder, lung	Briz et al., 2019; Kochel and Fulton, 2015; Wen et al., 2015; Zhang et al., 2015; Hlaváč et al., 2013; Chandra and Liddle, 2012; Kleberg et al., 2012; Chen and Tiwari, 2011; Tiwari et al., 2011; Russel et al., 2008; Kruh et al., 2007; Tian et al., 2005; Leggas et al., 2004; Lee et al., 2000
MRP5 / ABCC5	cornea, skeletal muscle, heart, brain, stomach, testis, lung, many healthy tissues	breast, breast cancer with bone metastasis, pancreatic, colon, lung, head and neck, ovarian, bladder, thyroid, esophageal, stomach, kidney, colorectal, cholangiocarcinoma, leukemia	Briz et al., 2019; Lal et al., 2017; Krajinovic et al., 2016; Chen et al., 2015; Wang et al., 2014; Hlaváč et al., 2013; Sodani et al., 2012; Partanen et al., 2012; Zhang et al., 2011; Karla et al., 2009; Hagmann et al., 2009; Calatozzolo et al., 2005; Dazert et al., 2003; Oguri et al., 2000
MRP6 / ABCC6	liver, kidney, pancreas, lung, thyroid, salivary gland, intestine	hepatocellular carcinoma, cholangiocarcinoma, lung, colorectal, kidney cancer, thyroid carcinomas, renal cancer	Briz et al., 2019; Eadie et al., 2019; Zhang et al., 2015; Beck et al., 2005; Belinsky et al., 2002
MRP7 / ABCC10	skin, colon, testes, pancreas, ovary, salivary glands	lung, colon, leukemia, hepatocellular, esophageal, colorectal, thyroid, breast	Briz et al., 2019; Balaji et al., 2016; Hlaváč et al., 2013; Borel et al., 2012; Tiwari et al., 2011; Bessho et al., 2009; Hopper-Borge et al., 2009; Wang et al., 2009; Oguri et al.,

			2008; Hopper-Borge et al., 2004; Dabrowska and Sirotnak, 2004; Hopper et al., 2001
MRP8 / ABCC11	liver, testes, breast, brain, placenta, mammary gland, prostate	breast, melanoma, lung, pancreatic, prostate, colorectal	Briz et al., 2019; Oba et al., 2016; Tsuchiya et al., 2016; Zhang et al., 2015; Wang et al., 2014; Yamada et al., 2013; Hlaváč et al., 2013; Toyoda and Ishikawa, 2010; Zhou et al., 2008; Oguri et al., 2007, Chen et al., 2005
ERCC1	Cells: nucleotide excision and repair pathway, a part of the cellular response to DNA damage	lung, bladder, breast, ovarian, cervical, esophageal, pancreatic, gastrointerstinal, colorectal	Anuja et al., 2019; Piljić Burazer et al., 2019; Dai et al., 2019; Lin et al., 2019; Mesquita et al., 2019; Takemoto et al., 2019; El Baiomy and El Kashef, 2017; Ryu et al., 2017; Kim et al., 2017; Du et al., 2016; Choueiri et al., 2015; Deng et al., 2014; Muallem et al., 2014; Steffensen et al., 2009; Wei et al., 2008; Kim et al., 2008; Bellmunt et al., 2007; Cobo et al., 2007; Kwon et al., 2007; Moore Joshi et al., 2005; Reed, 2005; Shirota et al., 2001; Metzger et al., 1998

MDR1/ABCB1, Adenosine Triphosphate - Binding Cassette Subfamily B Member 1; MRP1 / ABCC1, Adenosine Triphosphate - Binding Cassette Subfamily C Member 1; MRP2 / ABCC2, Adenosine Triphosphate - Binding Cassette Subfamily C Member 2; MRP3 / ABCC3, Adenosine Triphosphate - Binding Cassette Subfamily C Member 3; MRP4 / ABCC4, Adenosine Triphosphate - Binding Cassette Subfamily C Member 4; MRP5 / ABCC5, Adenosine Triphosphate - Binding Cassette Subfamily C Member 6; MRP6 / ABCC6, Adenosine Triphosphate - Binding Cassette Subfamily C Member 6; MRP7 / ABCC10, Adenosine Triphosphate - Binding Cassette Subfamily C Member 10; MRP8 / ABCC11, Adenosine Triphosphate - Binding Cassette Subfamily C Member 11; ERCC1, Excision Repair Cross-Complementation Group 1

Gazzaniga et al., have divided patients with bladder, CRC, BC, NSCLC, gatric, urothelial, ovarian, esophageal and head and neck tumors into “resistant” and “sensitive” groups based on their CTC chemoresistance-profile. Resistance prediction was reached in 95% of patients in progressive disease based on molecular sensitivity assay. They had also found the correlation between molecular test and chemotherapy response prediction in 98% of patients. In this publication Adenosine Triphosphate - Binding Cassette Subfamily C Member 4 (MRP4), Adenosine Triphosphate - Binding Cassette Subfamily C Member 5 (MRP5) and Adenosine Triphosphate - Binding Cassette Subfamily C Member 7 (MRP7) expression revealed resistence to anthracyclines and other ordinarily used chemotherapeutics. This publication had determined molecular CTC profile of drug resistance and chemotherapy response forecasting regardless of tumor type or disease stage. (Gazzaniga et al., 2010a) Moreover, the same group published another article about molecular markers in CTC from metastatic CRC patients in the same year. The analysis showed patients receiving oxaliplatin and 5-fluorouracil (as specific MRP5 substrates) who expressed MRP5 had shorter PFS and higher progression rate. (Gazzaniga et al., 2010b)

MRP transporters have been tested in CTC of metastatic BC patients, too (Gradilone et al., 2011). These patients have been divided into “drug sensitive“ and “drug resistant“ group based on specific MRP transporters expression profile. This paper agreed with previous results, shorter PFS had been observed in “drug resistant“ in comparison with “drug sensitive“ patients. Furthermore, detailed analysis of different MRPs expression and survival was provided. This examination showed patients who expressed two or more MRPs in CTC had shorter PFS than the ones who’s expressed one or none MRPs. This publication has supported the opinion that MRPs overexpression in CTC may be a poor response predictor and it is associated to shorter PFS. (Gradilone et al., 2011)

In a work published by Abdallah et al. metastatic CRC patients receiving irinotecan-based therapy underwent Adenosine Triphosphate - Binding Cassette Subfamily C Member 1 (MRP1) and MRP4 expression analysis in CTC, primary tumor and metastasis. This publication proposed CTC as the best tumor compartment for gathering reliable information on resistance to anticancer drugs in metastatic CRC.

Several examinations have been performed in this study showing interesting results for CTC. In the survival analysis, patients with MRP1 expression in CTC had shorter PFS and less median number of days for disease progression than those who did not. MRP1 positive CTC were then associated with KRAS mutation. It has been found that 75% of patients whose CTC expressed MRP1 had also KRAS mutation whereas patients with CTC without MRP1 expression had mostly wild-type KRAS. Another interesting analysis was performed in the case of MRP4 expression in CTC, primary tumor and metastasis. Although PFS was longer in the case of patients who expressed MRP4 in CTC and metastasis, inverse results were obtained in the case of primary tumor. From these data, CTC - MRP4 in the relation to PFS was a statistically significant result. This study recommended CTC as a marker for therapy selection. (Abdallah et al., 2016)

Except the ABC transporters, excision repair cross-complementation group 1 (ERCC1) gene expression has been measured in CTC in various tumor types including BC, lung or ovarian cancer. Das et al. measured expression level of ERCC1 in CTC of metastatic NSCLC patients who were treated with platinum-based therapy. Results of this analysis showed longer PFS was associated with lack of ERCC1 expression and vice versa. The study also revealed decreased PFS in CTC of patients who displayed increased expression of cytokeratin. This study concluded expression of ERCC1 in CTC correlates with PFS of these patients. (Das et al., 2012)

One of the most important studies has been published by Kuhlmann et al. who tested ERCC1 expression in CTC and PT of patients with ovarian carcinoma. This paper constituted CTC with ERCC1 expression as an independent predictor for OS and PFS. It had been discovered CTC with expression of ERCC1 at primary diagnosis may be considered as independent predictor of platinum resistance whereas its expression in analogous primary tumors predicted neither platinum resistance nor prognosis. CTC have become even more promising marker for patients with ovarian cancer. (Kuhlmann et al., 2014)

Ovarian cancer study of Chebouti et al. continued on previous publication by ERCC1 expression analysis of CTC in patients with ovarian cancer. They have

found a correlation between CTC expressing ERCC1 after chemotherapy and platinum resistance, shorter PFS and OS. It has been also observed that CTC positive for ERCC1, persisting in the circulation, indicated a poor post-chemotherapeutic outcome. CTC have been advocated as useful marker for platinum-based therapy monitoring and outcome. (Chebouti et al., 2017)

CTC of BC patients were also tested for several chemoresistance associated markers, including ERCC1 before and also after neoadjuvant therapy. A ratio of patients who expressed ERCC1 was lower before therapy in comparison to post-therapy samples, 63% and 72%, respectively. Surprisingly, 50% of the ERCC1 positive CTC post- therapy samples were positive for ALDH1 and at least for one another EMT marker. Additionally, Bredemeier et al., has revealed association between expression of ERCC1 alone or in combination with aurora kinase A (AURKA) gene and therapy failure. These results indicate ERCC1 positive CTC are able to survive treatment regimens and may represent therapy-resistant population of cancer cells. They may be a valuable source of information for secondary treatment decision. (Kasimir-Bauer et al., 2016; Bredemeier et al., 2016)

2. Thesis Aims:

Doctoral thesis is focused on liquid biopsy – CTC examination and its translational research in oncological therapy personalization.

The aims of this thesis were CTC enrichment and molecular characterization and can be summarized into following points:

- To set- up size- based enriched CTC *in vitro* cultures of cancer patients CTC in various treatment process stages; before therapy, undergoing chemotherapy, radiotherapy and biological treatment (e.g. anti – HER2), which is the prerequisite of successful molecular characterization of CTC in different solid tumor cancer types

- To evaluate cytomorphology of CTC with focus on cytomorphological changes of CTC during ongoing therapy (e.g. chemotherapy, radiotherapy, anti- HER2 treatment) in different cancer types

- To analyse gene expression profile of tumor-associated, stem cell-like and chemoresistance-associated genes in CTC in early and metastatic BC patients undergoing NACT with respect of CTC population heterogeneity under the chemotherapy pressure

- To assess prognostic and predictive potential of CTC based on molecular data correlated to the observed clinicopathological criteria in eBC patients

- To compare characteristics of primary tumor and CTC in BC patients undergoing NACT with respect of evolution of CTC in the mean of and HER2 status

3. Materials and Methods:

3.1. Patients

All of the patients' subsets are described in the corresponding articles, but in short: Publication I (page 212) – 20 eBC patients; Publication II (page 238) - 167 BC patients; Publication III (page 244) – 8 patients with various cancer diagnosis (4 patients BC, 2 CRC, 1 CRC + prostate and 1 lung cancer patient); Publication IV (page 261) – 40 ovarian cancer (CaOV) patients; and Publication V (page 269) – 118 CaOV patients were included into the studies.

All of the studies were performed in accordance with Declaration of Helsinki. Signed informed consent form had been obtained from each patient before start of any clinical procedure and patients' clinical data collection related to particular study.

Group 1 – Publication I (page 212)

Article: Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy (study of eBC patients)

The second paper studied 20 eBC patients. Patients underwent peripheral blood withdrawals for CTC analysis in regular intervals at 4 main time points: before NACT, during NACT and/or before and after surgery; and CTC monitoring continued for several patients also in long-term period after operation. In the cases NACT consisted of anthracyclines (AC) and/or taxanes (TAX) administration, CTC analysis were usually performed before the first AC cycle, before the first TAX cycle and before the last TAX cycle.
Other clinicopathological characteristics of patients who were enrolled into this study are described in Table 10.

Table 10: Detailed clinicopathological characteristics and therapy regimen of BC patients included in the Publication I.

Patients number	Age at the time of dg	Stage before therapy	Histology Ki67 (%)	Receptors from biopsy (%)	Regimen of NACT	Notices	Stage post-surgery	Histology Ki67 (%) post-surgery	Receptors post-surgery (%)
1	37	T2N1	IDC, G3, Ki 40	ER90, PR60, HER2-	4xAC-4xD	BRCA1 mutation	T2N2	IDC, G3, Ki 10	ER70, PR10, HER2-
2	43	T2N1	IDC, G1, Ki 50	ER100, PR50-60, HER2-	4xAC-4xD		T1aN0	IDC, Ki 30, G?	ER20, PR0, HER2-
3	71	T2N1	IDC, G3, Ki 40	ER100, PR60, HER2+	3xFEC-3xDH		T1cN0	IDC, G3, KI 3	ER100, PR100, HER2+
4	33	sin T1cN1 dx. T1aN0	IDC, G3, Ki 40	ER50, PR5, HER2+	4xAC-4xDH	CHEK2 mutation	sin. T1aN0 dx. pCR	IDC, G2, Ki 1	ER75, PR5, HER2-
5	39	T2N1	NST, G2, Ki 20	ER80, PR90, HER2+	4xAC-4xDH		T1cN1mi	NST, G2, Ki 10	ER70, PR30, HER2+
6	38	T2N0	IDC, G2, Ki 90	ER0, PR0, HER2-	3xFEC-3xD		pCR		

7	31	T2N1	IDC,G3,Ki 75	ER50,PR10, HER2-	4xAC, 1xD, 9xT	dg during lactation	pCR		
8	36	T1cN1	IDC,G3,Ki 55	ER0, PR5, HER2+	4xAC-4xDH		T1aN0	IDC,G3,Ki 5	ER0, PR0, HER2+
9	31	T2N1	NST,G3,Ki 70	ER50, PR70, HER2+	5xA	dg 18.w. of pregnancy 4x DH adjuvantly	T1cN0	NST,G1,**Ki 60**	ER0, PR0, HER2+
10	36	T2N0	NST,G3,Ki 75	ER0, PR2, HER2- classified as TNBC	3xFEC-3xD		T2N0	NST,G3, **Ki 95**	ER0, PR2, HER2- classified as TNBC
11	31	T2N1		ER5, PR7, HER2-			T1aN0		

			IDC,G3,Ki 80	classified as TNBC	4xACdd-4xD			IDC,G3,Ki 20	ER0, PR0, HER2-
12	33	T2N1	NST, G3, Ki 50	ER0, PR10, HER2+	4xACdd-1xDH, 9xTH		T2N1a(1/14)	NST, G3, Ki 20	ER0, PR0, HER2+
13	41	BC: T2N1	IDC,G3, Ki 100	ER0, PR0, HER2-	2xFEC75, 5xCBDCA+paklitaxel	vs BRCA1+	T1cN0	Metaplastic ca, G3, Ki50	ER5, PR1, HER2-classified as TNBC
		Ovary IIIc	Serous Ca,G3	ER75			OC: T3bN1	OC: same	
14	41	T1cN1	IDC, G3, Ki 50	ER80-90, PR20-25, HER2-	4xAC-4xD		T2N3	IDC, G3, **Ki 50**	ER100, PR25, HER2-
15	39	T1mN1	NST, G2, Ki 30	ER90, PR5, HER2-	4xAC-1xD, 9xT	dg during lactation	T2N1c	NST, G3, Ki0	ER100, PR10, HER2-
16	44	T1cN0	IDC, G3, Ki40	ER0, PR0, HER2-	6xFEC		T1bN0	NST, G1, Ki5	ER0, PR0, HER2-

17	44	T2N1	Medullary, G3, Ki70	ER0, PR0, HER2-	4xAC-12xT		pCR		
18	52	T2N1	IDC, G3, Ki50	ER100, PR50-75, HER2-	4xAC-4xD		T2N1	NST, G2, Ki5	ER100, PR1, HER2-
19	40	T2mN3	IDC, G3, Ki 60	ER0, PR0, HER2-	1xACdd, 3xAC,12 xT	BRCA1 mutation	Died before surgery		
20	40	T1cN0	IDC, G3, Ki 95	ER0, PR0, HER2-	3xFEC-9xT		pCR		

Group 2 – Publication II (page 238)

Article: Molecular characterization and heterogeneity of circulating tumor cells in breast cancer (study of BC patients with various disease stages)

The published research studied 167 patients who were diagnosed with BC. Patients included in this study were in different stage of the disease and were candidates for surgery, surgical diagnostics, or with planned or applied chemotherapy regimens. Clinicopathological characteristics of patients who were enrolled into this study are presented in Table 11. While most of the patients took part single blood withdrawal, regular monitoring was performed on 20 patients with respect on HER2 and ESR CTC status.

Table 11: Clinicopathological characteristics of BC patients included in the Publication II.

	N	(%)		N	(%)		N	(%)
Stage			**Histopathological features**			**Grading**		
0	3	2	Benign	2	01.7	G1	7	11.8
IA	45	30	DCIS	9	07.6	G2	24	40.6
IIA	64	42.7	LCIS	1	0.85	G3	28	47.4
IIB	20	13.3	IDC (NST)	76	65.6	**Menopausal status**		
IIIA	13	08.7	ILC	14	11.86	Premenopausal	65	39.39
IIIB	1	0.67	Mixed	16	13.6	Menopausal	18	10.9
IIIC	4	2.67	**HR and HER2 status**			Postmenopausal	82	49.7
Nodal involvement			HR+ HER2+	16	11.7	**Tumor size**		
N0	56	56.5	HR- HER2+	7	05.1	T1	63	61.1
N1	37	37.3	HR+ HER2-	91	66.4	T2	36	34.9
N2	6	6	HR- HER2-	23	16.8	T3	4	03.8

Group 3 – Publication III (page 244)

Article: Circulating tumor cells: what we know, what do we want to know about them and are they ready to be used in clinics? (study of case reports)

Publication no. 3 was published as selection of detailed case reports. Eight patients diagnosed with BC, CRC, prostate and lung cancer were included in this study for

long-term examination of CTC presence and their characteristics. Patients' tumor characteristics are showed in Table 12 which includes also staging and histology before and after surgery.

Table 12: Pre- and post- surgery patients' tumor characteristics included in Publication III

Cancer type / Age in the time of diagnosis	Staging before / after surgery	Pre-operative histology	Post-operative histology
BC / 34	b:T2N1M0; a: T2N1aM0	IDC, G3, TNBC, Ki67: 70%	Metaplastic carcinoma, G3, TNBC, Ki67: 70%
BC / 44	b: T1cN1M0; a: T1bN0(0/5)M0	ICD,G3, TNBC, Ki67: 40%	NST, G1, ESR10, PGR0, HER2NA, Ki67: 5%
BC/ 29	b: NA; a: pT1c multi(3) pN0 (0/2) M0	NA	TNBC, G3, Ki67: 60%
BC / 39	b: T2N1; a: T1cN1mi(2m/8)M0	IDC, G2, ESR80, PGR90, HER2+, Ki67: 20%	G2, ESR70, PGR30, HER2+, Ki67: 10%
CRC / 35	b: NA; a: pT2pN0(0/10)M0	NA	Tubular adenocarcinoma of rectum, G2, RAS?
CRC / 50	b: NA; a: pT2pN0(0/12)M0	NA	Adenocarcinoma of rectum, G2, RAS?
Lung / 47	b: NA; a: NA	NA	NA
CRC + PC / 57	b: NA; a: CRC: pT3pN0(14)M0, PC: T3bN0-1	NA	NA

Group 4 – Publication IV (page 261)

Article: Molecular characterization of circulating tumor cells in ovarian cancer. (molecular study of CaOV CTC)

In the last presented study, 40 women patients were involved. All of them were diagnosed with ovarian carcinoma in different stages and were also surgery or surgical diagnostic candidates.

Group 5 – Publication V (page 269)

Article: The added value of circulating tumor cells examination in ovarian cancer staging. (study of CaOV CTC value)

In total 118 women diagnosed with CaOV in various stages were included in the study. All patients were candidates for surgery or surgical diagnostics and their detailed features are showed in Table 13.

Table 13: Tumor characteristics of patients group included in Publication V

Patients characteristics						Total patients (N)
FIGO stage		**Histology**		**Grading**		118
IA	5	Serous	81	1	1	
IC	14	Serous/borderline	4	2	19	
IIA	1	Non serous (e. g. mucinous)	6	3	52	
IIB	2	Clear cell type	3	4	46	
IIC	4	Endometrioid	1			
IIIA	3	Undifferentiated	2			
IIIB	12	NA	21			
IIIC	69					
IV	8					

3.2. Methods

Patients' blood withdrawal as a part of liquid biopsy examination was followed by blood processing within 24 hours. After CTC enrichment, cytomorphology and gene expression analysis were applied for detailed CTC characterization.

3.2.1. CTC enrichment and cultivation of CTC

Patients' blood withdrawal as a part of liquid biopsy examination was followed by blood processing within 24 hours. After CTC enrichment, cytomorphology and gene expression analysis were applied for detailed CTC characterization.

Enrichment of CTC was done by size-based separation protocol at room temperature. Until the blood was processed, it was kept at 4-8ºC (temperature in the fridge). Approximately 6-8ml (volume was dependent on each study blood) of peripheral blood was filtered using Metacell® device (Metacell s.r.o., Czech Republic). (Figure 18) Cells captured on the filter were either cultured *in vitro* or stored for later gene expression studies.

After whole peripheral blood filtration, membrane with captured cells was washed by RPMI media (SIGMA-ALDRICH, USA) and placed into cultivation plate. 4 ml of RPMI media supplemented by fetal bovine serum (FBS) - 10% (SIGMA-ALDRICH, USA), Amphotericin B (SIGMA-ALDRICH, USA) and penicillin-streptomycin (SIGMA-ALDRICH, USA) antibiotics to avoid contamination were added on the membrane. The cells were cultured in vitro under standard conditions (37oC, 5% CO2) for 3-5 days. (Figure 18) Long-term cultivation was performed in the cases of massively growing cancer cells.

In the case of enriched cells storage, the cells with the membrane were directly after the filtration put into the 600 µl of Buffer RLT (QIAGEN, Germany) + β-mercaptoethanol (VWR, USA) and stored at -20°C.

Figure 18: The shortened protocol of CTC enrichment by Metacell. Cells which are bigger than 8 µm are caught on the membrane. After washing (RPMI medium), plastic ring keeping the membrane is then separated from tube and placed into the culturing wells. After cultivation (3-5 days), the membrane can be moved out of the plastic rings and placed directly on the microscopic slide. Cells undergo cytomorphological and/or molecular-genetics evaluation. (adapted from Bobek and Kolostova, 2018)

3.2.2. Cytomorphological analysis

After 3-5 days of *in vitro* cultivation, cytomorphological analysis of cells by fluorescent microscope was performed. Nucleus and cytoplasm of viable cells were stained by vital fluorescent dyes NucBlue® Live ReadyProbes® Reagent (Thermo Fisher Scientific, USA) and Celltracker™ Green CMFDA Dye (Thermo Fisher Scientific, USA), respectively. Stained cells were captured (magnification x40) by C software, Olympus IX51 fluorescent microscope with built in camera (Olympus U-RFL-T power supply unit) and identified using standardized cytopathologic criteria for CTC, which include: nuclei larger than 10µm, proliferation, presence of tridimensional cell-sheets, high nuclear/cytoplasmic ratio, prominent nucleoli, irregular nuclear membrane, visible cytoplasm, cells size above 15 µm. After cytomorphological analysis, cells captured on the membrane (membrane fraction) and meanwhile growing under the membrane (bottom fraction) were both disrupted by 600 µl of Buffer RLT + β-mercaptoethanol and stored at -20 ºC, respectively.

3.2.3. Gene expression analysis

White blood cells enrichment from whole peripheral blood

In total, 800 µl of Buffer EL (Qiagen, Germany) and 200 µl of peripheral blood were mixed together and incubated for 10-15 min on ice. After centrifugation of this suspension at 400 x g for 10 min at 4ºC supernatant was removed and discarded. Pellet of the cells was resuspended with 400 µl of Buffer EL by pipetting and this mixture was centrifuged at 400 x g for 10 min at 4ºC. Supernatant was completely removed and discarded. This procedure was completed by adding 600 µl of Buffer RLT supplemented by β-mercaptoethanol to the cells pellet. This suspension was mixed by pipetting and stored at -20ºC until RNA/DNA was isolated.

RNA isolation and reverse transcription reaction

All of the samples for RNA isolation were stored in Buffer RLT + β-mercaptoethanol solution. After defrosting (if there were stored in freezer, at -20ºC), RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation. Particular fractions of cells were analysed (1. white blood cells (WBC), 2. enriched cells (virgin CTC), 3. cultured cells on the membrane (membrane fraction CTC) and 4. cultured cells invading the membrane and setting up a colony under the membrane (bottom fractions).

Sample types differed in studies. Concentration of RNA was measured by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA) was used for cDNA synthesis. Reaction components were added according to manufacturing instructions as showed in Table 14.

Table 14: Instructions for preparing reverse transcription reaction mix (per 20 µl reaction)

Component	Volume/Reaction (µl)
2X RT Buffer	10
20X RT Enzyme Mix	1
Sample	Up to 9
Nuclease-free H_2O	To 20
Total per Reaction	**20**

Tubes with reaction mix were centrifuged and placed to the thermal cycler (ELISABETH PHARMACON, spol. s r.o., The Czech Republic). Conditions in the thermal cycler were set according to manufacturing instructions as shown in Table 15.

Table 15: Instructions of optimized conditions for High-Capacity RNA-to-cDNA™ Kit

Step	Temperature (°C)	Time (min)
Step 1	37	60
Step 2	95	5
Step 3	4	∞

Analysis of gene expression

After the reverse transcription reaction, differences between particular fractions were detected by qPCR analysis of tumor- and/or stem-cells like- and/or resistance-associated genes. For gene expression monitoring in the samples TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used. Conditions in the cycler were set according to manufacturing instructions as shown in Table 16 and monitored genes are listed in Table 17.

Table 16: Instrutions to program the thermal-cycling conditions for the real-time PCR

Step	**Temperature** (°C)	**Time (sec)**	**Number of cycles**
*UNG Incubation**	50	60	1
Enzyma activation	95	20**	1
Denature	95	1	40
Anneal/Extend	60	20	

*Optional, for optimal UNG activity
**Enzyme activation can be up to 2 minutes. The time should not cause different results.

Table 17: List of Taqman assay codes for gene expression profiling of tumor associated genes and genes associated with chemoresistance.

Name	Assay	Hs number
MDR1	ABCB1	Hs00184500_m1
MRP1	ABCC1	Hs01561502_m1
MRP2	ABCC2	Hs00166123_m1
MRP4	ABCC4	Hs00988717_m1
MRP5	ABCC5	Hs00981087_m1
MRP7	ABCC10	Hs00375701_m1
ActB	ACTB	Hs01060665_g1
CD24	CD24	Hs02379687_s1
CD44	CD44	Hs01075861_m1
CD68	CD68	Hs02836816_g1
EpCAM	EPCAM	Hs00158980_m1
Her2	ERBB2	Hs01001580_m1
ERCC1	ERCC1	Hs01012158_m1
ESR	ESR1	Hs00174860_m1
KRT18	KRT18	Hs01920599_gH
KRT19	KRT19	Hs01051611_gH
MUC1	MUC1	Hs00159357_m1
PGR	PGR	Hs01556702_m1
CD45	PTPRC	Hs04189704_m1
MGB	SCGB2A2	Hs00935948_m1

Results of genes expression were processed, evaluated and charted by GenEx software (MultiD Analyses AB). Several groups had been set in measured data which were based on patients, fraction type (WBC, virgin CTC, membrane fraction CTC and bottom CTC fraction), HR and HER2+ primary tumor status, results of cytomorphology and genes expression analysis and monitored genes. Beta-actin

(ActB) gene was selected as reference gene due to its stability and enough amount presence in all of the tested samples. Normalization to this reference genes was performed which corresponds to ΔCq calculation. Relative quantities process was the next step of pre-processing. Quantity our samples were given to relation with WBC fraction of corresponding withdrawal. This step corresponds to ΔΔCq calculation. Relative RNA amounts are showed in $\log_2$ values. Data in particular groups were compared visually in bar graphs by Mann-Whitney test (2-tailed). Hierarchical clustering of samples and genes was combined displaying the measured intensities in a heat map. (GenEx User Guide; Bredemeier et al., 2017)

4. Results and comments

The research accompanying this PhD thesis was aimed to CTC in various cancer diagnoses with main focus on breast cancer. Peripheral blood samples were withdrawn from cancer patients and CTC were enriched by size-based separation. Further processing of CTC included their microscopic and molecular analyses that confirm their presence and provide detailed characterisation.

4.1. Breast cancer

The published data of **Publication I** (page 212) report the dynamics of CTC changes in 20 patients diagnosed with early BC. This CTC status was monitored for a period of the 6-24 months. Enrolled patients had received neoadjuvant chemotherapy (NACT) before surgery and their detailed clinicopathological characteristics are showed in Table 10. CTC were analysed cytomorphologically and by gene expression. Gene expression analysis was provided for tumor- and chemoresistance-associated genes and stem cell-like markers. All together 20 genes were tested per CTC-positive sample. Individual long-term monitoring of medical treatment and CTC status was performed in all of the 20 patients.

Tumor response for NACT was monitored by bimanual palpation and ultrasound examination approximately at the time of blood withdrawal for CTC examination. According to these results tumor regression / progression were observed and responders / non-responders were identified. Tumor mass regression was assessed as very significant (response rate 3, more than 50% regression), moderate (response rate 2, 50% regression), minimal (response rate 1, less than 50% regression) and no regression observed (response rate 0).

Subsequently, correlation between tumor regression and CTC was studied. Significant effect of AC and TAX was reported in 40% and 23% of patients where CTC positivity was detected in 87.5% and 75% of patients, respectively. Among non-responders, 85%-100% CTC positivity was detected. (Table 18) Based on the data on tumor volume regression, we may assume that tumor regression was not associated with CTC positivity in our patients' sample receiving NACT.

Table 18: Overview of tumor volume regression during NACT in Publication I

	CTC	Regression of tumor volume (Response rate - RR)							
	Positivity (%)	**RR = 0 (%)**	**CTC in group RR =0 (%)**	**RR = 1 (%)**	**CTC in group RR =1 (%)**	**RR = 2 (%)**	**CTC in group RR =2 (%)**	**RR =3 (%)**	**CTC in group RR =3 (%)**
Before Therapy	17/20 (85)	-	-	-	-	-	-	-	-
After AC	16/18 (88)	1/20 (5)	1/1 (100)	7/20 (35)	6/7 (85)	4/20 (20)	3/4 (75)	8/20 (40)	7/8 (87.5)
After TAX (before surgery)	13/18 (72)	3/17 (18)	3/3 (100)	9/17 (53)	7/8 (87.5)	1/17 (6)	1/1 (100)	4/17 (23)	3/4 (75)

The **Publication II** (page 238) is focused on CTC - testing in 167 BC patients. They were divided into several groups based on their disease status and clinicopathological criteria. These parameters were detected by standard medical examination of primary tumor.

Four BC case reports in the context of CTC are described in **Publication III** (page 244). CTC presence and character have been monitored for long-term (6-24 months). Most of the blood withdrawals were evaluated as CTC-positive. CTC results were evaluated in relation to the clinical status of each individual and their future perspectives.

Following questions were asked regarding CTC studies in BC patients

Does CTC character differ from primary tumor features?

Does the cancer cells morphology and molecular profile develop in the course of disease?

Are the molecular markers of the disease changing during treatment process?

Is the evolution of BC primary tumor molecular heterogeneity detectable by CTC molecular characterization?

Do CTC reflect real-time disease status?

Could CTC contribute to assess disease prognosis?

Does expression of chemoresistance associated genes on CTC indicate worse therapy response during NACT?

Results of BC research are described below for each scientific paper

Publication I (page 212)

CTC positivity was also evaluated in relation to particular tumor types. We had found the highest CTC positivity in the peripheral blood of patients with ESR+ and HER2+ tumors corresponding to 90% and 91%, respectively. The lowest, 76% was detected in patients with TNBC primary tumors. (Table 19)

Table 19: CTC + samples proportions regarding primary tumor types

Primary tumor type	Primary tumor types occurence	CTC + samples at all	CTC + during NACT	CTC + at any time
ESR+	5/20	27/30 (90 %)	1/13 (8 %)	1/27 (4 %)
HER2+	6/20	38/42 (91 %)	12/15 (80 %)	18/38 (47 %)
TNBC	9/20	28/37 (76 %)	12/18 (67 %)	19/28 (68 %)

plus (+) represents positive

Consistence of ESR+, HER2+ and TNBC expression was also studied. Our results showed relatively low concordance between primary and CTC, 1/13 and 1/27 in the ESR+ tumor type throughout the whole monitoring type. Higher accordance between primary tumor and CTC was observed in HER2+ and TNBC tumors not only during NACT but also within whole monitoring period. (Table 19 and Table 20)

Table 20: Status of primary tumor and CTC in relation to tumor types and neoadjuvant chemotherapy

Primary tumor	CTC			
	ESR+	ESR-	HER2+	HER2-
	NACT - any time	NACT - any time	NACT - any time	NACT - any time
ESR+ HER2-	**1/13 (8 %) - 1/27 (4 %)**	12/13 (92 %) - 26/27 (96 %)	4/13 (31 %) - 7/27 (26 %)	**9/13 (69 %) - 20/27 (74 %)**
ESR- HER2-	0/18 - 0/28	**18/18 (100 %) - 28/28 (100 %)**	6/18 (33 %) - 9/28 (32 %)	**12/18 (67 %) - 19/28 (68 %)**
ESR+ HER2+	**0/10 - 5/26 (19 %)**	10/10 (100 %) - 21/26 (81 %)	**9/10 (90 %) - 13/26 (50 %)**	1/10 (10 %) - 13/26 (50 %)
ESR- HER2+	3/5 (60 %) - 3/12 (25 %)	**2/5 (40 %) - 9/12 (75 %)**	**3/5 (60 %) - 5/12 (42%)**	2/5 (40 %) - 7/12 (58 %)

plus (+) represents positive; minus (-) represents negative

Monitoring of CTC was performed in a regular manner from diagnosis through NACT to post-surgery follow-up period. As part of this process, real-time CTC characteristics were examined by gene expression analysis. It had been showed CTC features were changing not only in the case of tumor-specific characteristics but also in chemoresistance-associated features. Our results indicated that several epithelial markers and chemoresistance-associated genes expression during NACT had seemed to be linked with positive clinical effect of treatment. Surprisingly, CTC negativity was detected only in the cases of relevant clinical response (response rate as set by ultrasound) (RR=2 or 3), after AC (patient no. 2, 8 and 14) or TAX (patient no. 2, 5, 7, 13 and 17) therapy. On the contrary, CTC of patients with poor clinical response (RR=0 or 1) had usually higher expression of two or more chemoresistance-associated genes detected and number of these genes was increased throughout treatment. (Table 21)

Except chemoresistance-associated markers, other characteristics of CTC had been monitored. Changes of ESR and HER2 expression status during NACT were dynamic. Several different scenarios occurred. Higher HER2 gene expression level was detected only before NACT (patient no. 16), during therapy (patient no.12), in several peripheral blood withdrawals (patient no. 3, 4, 5, 6, 8, 9, 10) or in none

(patient no. 13, 14, 15, 17, 18, 19, 20) of them. (Table 21) On the other hand, ESR was detected in 4 CTC samples of 3 eBC patients (patient no. 7, 8, 12) in our research. (Table 21)

Table 21: Overview of status of CTC presence and characteristics and clinical effect of neoadjuvant chemotherapy.

Patient	CTC before NACT			Clinical effect of AC	CTC after AC			Clinical effect of TAX	CTC before operation (after TAX)			Effect of completed NACT
	YES (1)/ NO (0)	Genes with elevated relative RNA levels			YES (1)/ NO (0)	Genes with elevated relative RNA levels			YES (1)/ NO (0)	Genes with elevated relative RNA levels		
		TA- genes	CA-genes			TA- genes	CA- genes			TA- genes	CA - genes	
1	1	EPCAM, KRT19, MUC1	MRP1, MRP4, MRP5, MRP7	1	1	EPCAM, KRT19, MUC1, HER2, CD24		0	**not evaluated**	-	-	1
2	0	**CTC negative**	**-**	**3**	**1**	EPCAM, KRT29, MUC1, HER2	-	1	**0**	**CTC negative**	**-**	**3**
3	1	EPCAM, KRT19	MRP7	2	1	KRT19, HER2	MRP1, MRP7, ERCC1	1	1	EPCAM, HER2, KRT19	MRP2, MRP4	2
4	1	HER2, KRT19	-	**3**	**1**	HER2, KRT19, EPCAM,MUC1, CD24,CD44	MRP1, MRP5, MRP7, ERCC1	2	1	KRT19, CD24,CD44,	-	**3**

5	1	HER2, KRT19	**-**	**3**	**1**	KRT19, HER2, CD24	-	0	**0**	**CTC negative**	**-**	2
6	1	EPCAM, KRT19, HER2	MRP5	**3**	**0**	**CTC negative**	**-**	1	1	HER2, MUC1, KRT19,MGB, CD24,CD44,	MRP5, MRP7, ERCC1	**3**
7	1	EPCAM, KRT19	-	2	1	HER2, ESR1, KRT19, MUC1, CD24,CD44,	MRP1	**3**	**0**	**CTC negative**	**-**	**3**
8	0	**CTC negative**	-	2	1	HER2, KRT19, MUC1, CD24, ESR1	-	1	1	KRT19, MGB, CD24, HER2	MRP1, MRP5, MRP7	**3**
9	1	KRT19, CD24, HER2	-	**3**	**not evaluated**	-	-	no taxanes	1	EPCAM, HER2, KRT19, CD24	MRP1, MRP7	2
10	1	KRT19, CD24, HER2	MRP1, MRP4, MRP5, MRP7, ERCC1	1	1	HER2, KRT19, MGB, MUC1, CD24, CD44,	MRP1, MRP5, MRP7,	0	1	CD24, CD44, KRT19	MRP1, MRP4, MRP5, ERCC1	1
11	1	KRT19, CD24, HER2	MRP1, MRP5, MRP7	2	**0**	**CTC negative**	**-**	**3**	**1**	KRT18, KRT19, CD24	MRP1 MRP7	**3**

12	1	KRT19, MGB, ESR1, CD24, CD44,	MRP1, MRP4	1	1	KRT19, HER2, CD24, ESR1,	MRP1	1	1	CD24, CD44, KRT18, KRT19	MRP1, MDR1	1
13	1	KRT19, MGB, CD24, CD44	MRP1, MRP5, MRP7	1	**not evaluated**	-	-	**1**	**0**	**CTC negative**	**-**	**3**
14	**0**	**CTC negative**	**-**	1	1	KRT18, KRT19, CD24, CD44,	ERCC1, MRP1	1	**not evaluated**	-	-	0
15	1	KRT18, KRT19, CD24	MRP1, MRP4	0	1	KRT18, KRT19, CD24	MRP1, MRP7	1	1	KRT18, KRT19, CD24	-	0
16	1	KRT18, KRT19, MUC1, HER, CD24, CD44,	ERCC1, MDR1, MRP1, MRP5, MRP7	*3*	1	CD24, CD44, KRT18, KRT19, MUC1	ERCC1, MRP1, MRP2, MRP5	no taxanes	**not evaluated**	-	-	*3*

17	1	KRT18, KRT19, CD24, CD44,	MRP1, MRP4, MRP7	*3*	1	KRT18, KRT19, CD24	-	3	**0**	**CTC negative**	**-**	*3*
18	1	EpCAM, KRT18, KRT19, CD24	MRP1	1	1	KRT18, KRT19, CD24	MRP4	1	1	KRT18, KRT19, MUC1, CD44	MRP1, MRP2, MRP7	3
19	1	KRT18, KRT19, CD24, CD44,	ERCC1, MRP1	*3*	1	KRT18, KRT19, CD24, CD44,	MRP1, MRP7	3	1	KRT18, KRT19	MRP1, MRP2, MRP7	Died
20	1	KRT18, KRT19, CD24	ERCC1, MDR1, MRP1, MRP4	1	1	KRT18, KRT19, MUC, CD24, CD44,	MRP1	**3**	**1**	KRT18, KRT19, CD24, CD44	MRP4	**3**

Changes in epithelial and stem-cells like features were detected. Most of the patients exhibited exclusively CTC with higher levels of epithelial markers before NACT (KRT19, EpCAM, mucin 1 (MUC1), etc.). Increased stem cells-like (CD44, CD24) characteristics expression was observed in CTC during or after NACT therapy. There were patients with elevated stem cells-like markers expression also before NACT. (Table 21)

After NACT, the surgery had taken place and status of both disease and CTC was monitored in several patients. CTC were observed in 100% at different time points. Regarding CTC characteristics, in patients with ESR+ tumor type CTC with epithelial features were detected more frequently in comparison with stem cells-like. Although certain trends in disease behaviour were observed, development of both CTC and tumor tissue(s) was individual in each patient monitored. (Table 21)

Publication II (Page 238)

Overall result of CTC positivity examination in this study was 76% from the patients with BC. (Figure 19) Patients were the divided into groups based on their stage, histopatological characteristics, menopausal status, tumor size, nodal involvement, grade, hormonal receptors and HER2 status of primary tumor and therapy state.

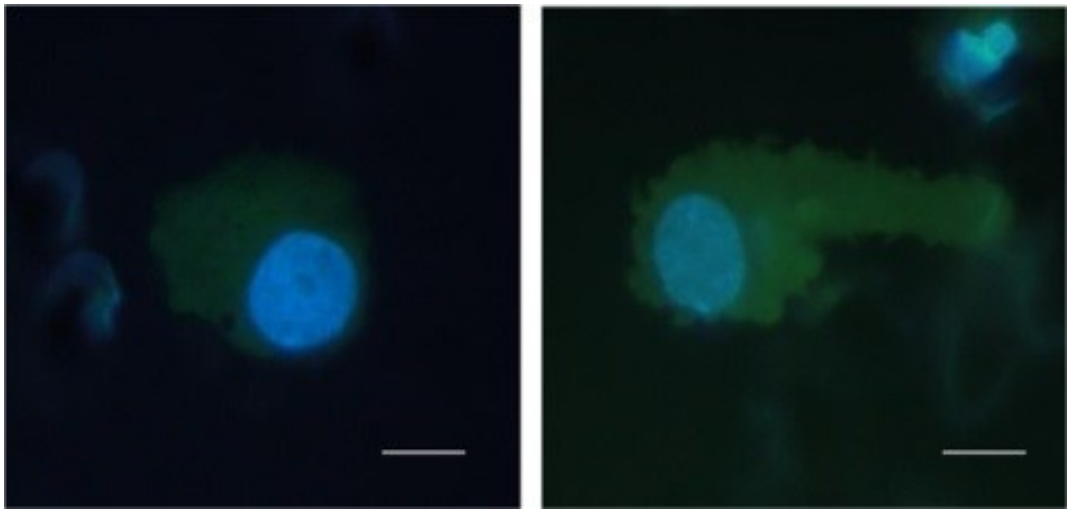

Figure 19: CTC enriched from peripheral blood of breast cancer patients after cultivation on the membrane after vital fluorescent staining with NucBlue™ and CellTracker™. Bar represents 10 µm (adapted from Jakabova and Bielcikova et al., 2017)

Regarding histopathological characteristics we had identified the lowest CTC positivity, 44.4%, in ductal carcinoma *in situ* (DCIS**)** patients' group. The highest percentage, 82%, was showed within grade 3 (G3) patients' group. The menopausal and premenopausal group showed 55.5% and 78.4% of CTC - positive samples, respectively. Impact of therapy on CTC presence had been also studied. We found high CTC occurrence in the groups before therapy and surgery which were 82.3% and 86.6%, respectively. Group of patients who were undergoing hormonal therapy showed 52.9% CTC positivity. CTC had been examined also in the relationship with primary tumor and HR and HER2 status. The highest CTC positivity was detected in HR- HER2+ and the lowest in patients with HR+ HER2- primary tumors corresponding 100% and 70.3%, respectively. In patients' groups with primary tumors characteristics as ESR+ PGR- and ESR+ PGR+, CTC were identified in 60% (9/15) and 73% (68/93) of tested samples, respectively. In ESR+ tumors CTC occurred in 96.7% (30/31) of samples.

The second part of this study was a regular long-term monitoring of 20 BC patients during the course of the disease. CTC samples were analysed by both cytomorphological and molecular examination. Gene expression tests have been focused on ESR and HER2 status of CTC. This analysis had revealed difference between primary tumor and CTC in ESR and HER2 expression. Although 12 of these patients had been diagnosed with TNBC, HER2 status changed from HER2- to HER2+ in at least four cases (33.3%). Similarly, 50% (3/6) of patients who had been initially HER2+ changed to HER2-. In the case of ESR status; it had been changed only from ESR+ to ESR- (3/3) in our patients' sample.

Publication III (page 244)

The results of the research in reported paper is processed in the form of case study reports. Patients' no. 1, 2, 3 and 4have been diagnosed with BC and their monitoring outcomes are dscribed below.

Patient No. 1 (TNBC; 34 years old, woman) underwent 3 blood withdrawals presented in this study. There were 2 CTC examinations during neoadjuvant treatment and 1 CTC analysis after primary tumor surgery. At the time of diagnosis gene expression of KRT18, KRT19, MUC1 and MRP1 increased in CTC. After AC

(doxorubicin + cyclophosphamide) treatment KRT18, KRT19, VIM, ALDH and MRP1, MRP7 and ERCC1 were elevated. Primary tumor grew from 28 mm to 32 mm (35 mm according to final histology) during neoadjuvant therapy. The last withdrawal was performed 1 month after surgery, increased level of CD44 and MRP1 was reported. None of the CTC examination had confirmed increased expression level of HER2 or HR.

Patient No. 2 (TNBC; 44 years old, woman) underwent long-term CTC monitoring. Before start of NACT following genes were increased in enriched CTC-fraction: KRT18, KRT19, MUC1 and MRP1. Surprisingly, elevated level of HER2 in CTC was also confirmed. Although after surgery there were still CTC detected, after radiotherapy treatment CTC were no longer present in peripheral blood of this patient. During adjuvant chemotherapy (capecitabine) treatment, patient was evaluated as CTC-positive again. This withdrawal had revealed CTC clustering and increased expression of mesenchymal markers, such as VIM and ALDH. Expression of HER2 was confirmed in CTC only before the therapy.

Patient No. 3 (TNBC; 29 years old, woman) was tested for CTC presence exclusively in the postoperative period (adjuvant setting (AS)). During AS the doxorubicin + cyclophosphamide – docetaxel (AC-D) treatment, CTC have expressed epithelial characteristics; in the follow-up period mesenchymal features were in prevalence. First withdrawal was provided at the start of AS (AC-D treatment) and EpCAM, KRT19, ESR and MRP1 gene expression was increased in the CTC fraction. During therapy, CTC expressed MUC1, KRT19, HER2 (not ESR anymore), MGB, MRP1 and MRP7, at the end of treatment epithelial characteristics and MRP1 with ERCC1 were increased. None of the HR or HER2 showed elevated expression. In the follow-up period, expression of mesenchymal genes started to be increased even though number of CTC had a decreasing tendency.

Patient No. 4 (BC - ESR+, PGR+, HER2+; 39 years old, woman) underwent 6 blood withdrawals for the purpose of CTC examination and all of them were evaluated as CTC positive. Although at the time of diagnosis two genes, HER2 and KRT19 were increased, after NACT (AC therapy) epithelial (EpCAM, KRT19, MUC1), mesenchymal (CD44), HER2 and chemoresistance-associated (MRP1, MRP5,

ERCC1) genes were increased. After another therapy with docetaxel + herceptin (DH), genes CD24, CD44, KRT19, ESR and MRP1 expressed elevated expression levels. Although CTC features were changing, size of the primary tumor diminished from 31x25 mm to 12x15 mm. During another treatment, firstly with tamoxifen + herceptin and later with tamoxifen alone, the patient generalized into central nervous system (CNS) and CTC expressed KRT18, KRT19, CD24, CD44, MRP1, Adenosine Triphosphate - Binding Cassette Subfamily C Member 2 (MRP2) and MRP5. Shortly after CNS metastasis diagnosis the patient died.

Comments and conclusion of BC research are described below

Several important aspects of CTC were taken into notice in this research. Microscopical evaluations, expression analysis of genes and detailed personalization had been studied to better understand their potential for early, advanced or metastatic BC patients.

Some of the researches had confirmed that CTC are an independent prognostic factor in BC. On the contrary several of them have found correlation between CTC count and positive lymph nodes or HER2 status of the primary tumor. (Bauer et al., 2018; Cherdyntseva et al., 2017; Riethdorf et al., 2017; Mego et al., 2017; Bidard et al., 2016; Van Dalum et al., 2016; Usiakova et al., 2014) Our study presented as **Publication II** (page 238) reported the lowest CTC positivity in DCIS carcinoma and in menopausal women. The highest CTC positive patients were observed in grade 3 of primary BC tumors and premenopausal BC patients.

Since the first step of CTC analysis is cytomorphogical evaluation, changes in CTC number and morphology may be the useful indicator of actual disease status and therapy efficiency. Previous studies have already confirmed CTC enumeration and morphological character in various part of treatment can be independent prognostic factor. They may help with patients' stratification and predictive biomarker for therapy selection in numerous cancer types. (Sundling and Lowe, 2018; Li et al., 2018; Riethdorf et al., 2017; Yan et al., 2017; Potdar and Lotey, 2015) In the **Publication III** (page 244) morphological dynamics of CTC was monitored and several individual cases were observed. CTC numbers have been decreasing (patient no. 3) or increasing (patient no. 2) throughout therapy. Other situation

occurred in the case of CTC - clustering and aggressive growth at the membrane (patient no. 1) which was supposed to unfavourable as the CTC in cluster have greater ability to metastasize compared with single CTC. (Cherdyntseva et al., 2017)

Our study that is reported as **Publication I** (page 212) is focused on patients treated with NACT. Several researches have confirmed CTC as an independent prognostic biomarker for these patients and it was also in accordance with our study. (Bidard et al., 2018; Riethdorf et al., 2017) During monitoring period we found CTC presence in all sampling points (not only during NACT but also after surgery and/or following therapy). These samples revealed persisting CTC (patient no. 8 and 17) or CTC-negative blood withdrawal (patient no. 6, 10 and 11) despite pCR achievement followed by NACT (patient no. 6 and 17). Indeed, CTC positivity after several months or years after primary diagnosis is not a rare event. Similar studies have confirmed their occurrence in blood 2 years after chemotherapy or 5 years after primary eBC diagnosis even without any clinical signs of disease recurrence. (Trapp et al., 2018; Bauer et al., 2018; Sparano et al., 2018)

Other important results had been obtained regarding HR and HER2 status of primary tumor presented in **Publication II** (page 238). These parameters are linked closely to the treatment choice based on primary tumor status. (Al-Mahmood et al., 2018; Hortobagyi et al., 2018; Bøttcher et al., 2018; Loibl and Gianni, 2017) In our patients samples the highest CTC positivity was detected in blood samples before surgery and/ or before therapy. The lowest CTC numbers were observed in patients during hormonal therapy. Although studies had confirmed lower CTC count after therapy in most cases, a decrease in CTC number is not clearly associated with TNBC type. (Cabel et al., 2017; Yan et al., 2017; Van Dalum et al., 2015) This attitude is consistent with our results as CTC positivity after neoadjuvant therapy was 77.7%

Apart from CTC presence examination, gene expression analysis of ESR and HER2 in CTC was performed. These results were then compared with primary tumor status. We found the lowest concordance status in patients with ESR+ BC diagnosis whereas higher concordance rate was observed in TNBC and HER2+ in our

patients' sample presented in **Publication I** (page 212). Our study reported as **Publication III** (page 244) also confirmed that the concordance between CTC and primary tumor is not always the case. There were several different situations that occurred; CTC of patients with TNBC had expressed neither HER2 nor receptors through all long term-monitoring (patient no. 1); partial accordance CTC and primary tumor at the time of diagnosis (patient no. 4) or discordance of CTC and primary tumor at the time of primary tumor characteristics determination (patient no. 3). Researchers from other groups demonstrated different concordance rates and found CTC were often negative or HER2 positive in their studies. Occurrence of HER2+ CTC in TNBC patients was not occasional. They had also revealed the highest discordance in ESR between CTC and primary tumor in various stage of BC. (Bittner et al., 2018; Brown et al., 2019; Jaeger et al, 2017; Aktas et al., 2017) It was found HER2+ CTC had usually prevailed during disease evolution. They were also detected in peripheral blood of patients who had been previously classified into HER2- CTC group. (Pierga et al., 2018; Agelaki et al., 2017)

Gene expression analysis in our study presented in **Publication II** (page 238) revealed that CTC status changed from HER2- to HER2+. This discordance between primary tumor tissue and CTC is not rare event. Frequency of discordance rates has not been clearly identified in bigger cohorts and remained individual. Routine long-term monitoring of BC heterogeneity from diagnosis through therapy and follow-up phase could be promising strategy for better outcomes achievement. (Aktas et al., 2018; Agelaki et al., 2017; Ellsworth et al., 2

CTC usually represent a heterogeneous sub-population of cells with different biological behaviour or ability to metastasize. CTC population undergoes changes during therapy. (Kaigorodova et al., 2018; Cherdyntseva et al., 2017) During long-term monitoring we found CTC character in terms of other genes expression had changed through therapy. Markers KRT18, KRT19, EpCAM, MUC1, CD24 and CD44 genes expression was monitored in CTC obtained from our patients enrolled into studies presented in **Publication I** (page 212) **and Publication III** (page 244). However, these changes were highly individual. Since at least one of the keratins was expressed in majority of our patients'samples, other important markers were

expressed differently in certain sampling points. Expression of EpCAM marker was evaluated as it is essential molecule for enrichment and detection process of CTC by several methods. (Sharma et al., 2018; Pugia et al., 2017) Whereas CTC of patients no. 6 and 18 showed higher EpCAM expression only in the first withdrawal or exclusively during NACT (patient no. 1), patient no. 10 demonstrated increased expression of this marker entirely in the fifth blood withdrawal (24 months after diagnosis) in **Publication I** (page 212). Another study confirmed that although CTC expressed epithelial features at the start of therapy, they changed to mesenchymal or stem cell-like markers till the next withdrawals (patients' no. 1, 3, and. 4) as presented in **Publication III** (page 244). Other researches had also supported the hypothesis of variable EpCAM expression in CTC and its loss had been considered as a result of dynamic changes during EMT. (Aaltonen et al., 2017; Bredemeier et al., 2017; Wang et al., 2017)

In regard to stem cells markers, CD24 and CD44 genes, expression on CTC we report differences between the different sampling times in **Publication I** (page 212). It has been found that undifferentiated tumor cells with $CD44^{+}/CD24^{-/low}$ gene expression profile play important role in cell proliferation, tumorigenesis, relapse and metastasis in BC patients. Approximately one-third out of all circulating cells shows this character. (Bai et al, 2018; Li et al, 2017; Zarzynska, 2017) Identification of these cells has been determined to circulate in the body throughout whole disease and may be helpful in metastasis recognition from early stages. (Dasgupta et al., 2017; Mansoori et al., 2017)

Regular examination of chemoresistance-associated markers in CTC-positive samples was provided as part of the **Publication I** (page 212). Based on this research, we could observe differences between responders and non-responders via CTC character. A minimum of chemoresistance-associated genes was expressed on CTC of responders. In the group of non-responders usually two or more of these markers were overexpressed. Detailed research also revealed that elevated MRP1 expression corresponded to the after - AC effect. We also detected significant progression-free survival rate difference between groups of patients with higher expression of more and less than four increased chemoresistance-associated markers in CTC. Studies published by other groups had showed similar results.

Increased expression of resistance-associated markers may be helpful in various aspects of disease monitoring. Whereas patients with drug resistant CTC profiles exhibited progressive disease, those with drug sensitive CTC characteristics had partial/complete response or stable disease. Moreover, chemoresistant CTC indicated significantly shorter PFS in comparison with the other group. Their predictive value has been also examined. CTC with higher expression of chemoresistance-associated genes were predictive of chemotherapy resistance. (Adamska et al., 2018; Ween et al., 2015; Eroglu et al., 2013)

4.2. Ovarian cancer

CaOV belongs to the most common and serious malignancies of the women reproductive system. In most cases, it is diagnosed in the advanced stage because early-stage disease is often asymptomatic and late-stage disease symptoms are nonspecific. This fact has seemed to be the cause of its high mortality. It had been found that only around 25% of these tumors are diagnosed by the time when tumor is still present in the ovary. The cure probability in the CaOV is getting worse with the disease spreads. (Smith, 2017; Doubeni et al, 2016)

In the studies of CTC in CaOV patients, cytomorphological together with gene expression analysis were provided. Together 56 and 118 CaOV patients in different stages were enrolled into this research presented as **Publication IV** (page 261) and **Publication V** (page 269), respectively.

Following questions were asked regarding CTC studies in CaOV patients

Is enrichment of CTC by MetaCell® effective enough for obtaining of viable CTC and representative heterogeneous CTC sample?

Could CTC examination significantly enhance in the CaOV staging process in patients with recurrent disease?

Is there a difference in molecular character of CTC developing under different environment conditions (e.g. chemotherapy, radiotherapy, *in vitro* culture)?

Results of CaOV research are described below for each scientific paper

In our patients' sample in **Publication IV** (page 261), 32 (58%) of them were assessed as CTC-positive and 24 (42%) were included in CTC-negative group based on cytomorphological examination. (Figure 20)

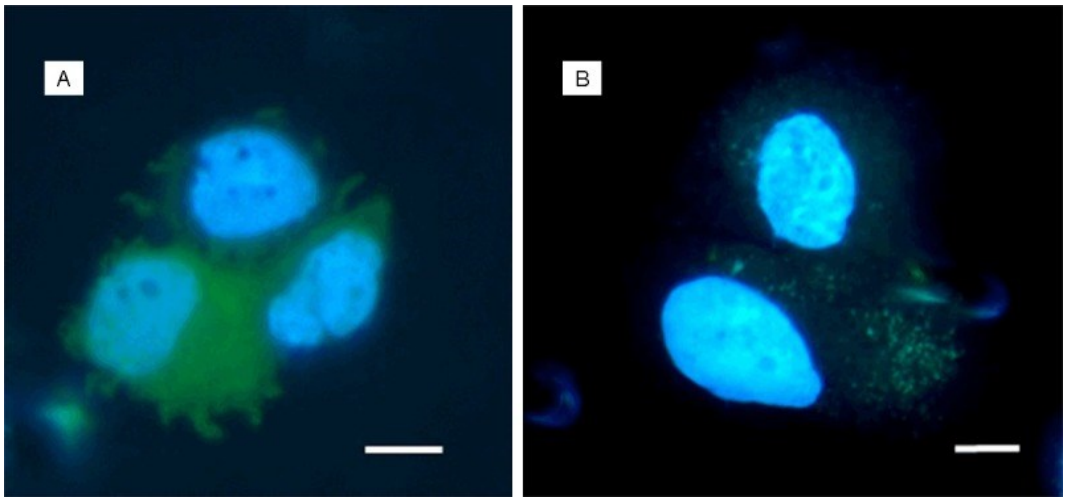

Figure 20: Cultivated CTC on the membrane from ovarian cancer patients after enrichment and short-term cultivation in publication IV. Cells were stained by NucBlue™ and CellTracker™ and captured by fluorescent microscope. The bar represents 10µm. (adapted from Kolostova et al., 2016)

Our results in **Publication V** (page 269) showed that 77 out of 118 (65.2%) CaOV patients, enrolled into our study, were evaluated as CTC-positive based on cytomorphological evaluation (Figure 21). Although no significant correlation between CTC and lymph nodes involvement was found in this study, in the case of more severe disease status (higher grade, worse FIGO stage) CTC were detected in more than 2/3 of the cases.

Out interest in **Publication V** (page 269) had been also focused on correlation of CTC presence with other patients'clinicopathological characteristics. Patients with ascites, peritoneal carcinomatosis and residual disease had been evaluated as CTC positive in 73.6% (39 out of 53), 70.5% (43 out of 61) and 75.0% (42 out of 56) in our study.

Another important CaOV marker CA125 had been studied in the context of CTC presence in **Publication V** (page 269). We concluded elevated level of CA125 could be a reliable marker of peritoneal spread and CTC presence could be a significant marker of haematogenous CaOV spread.

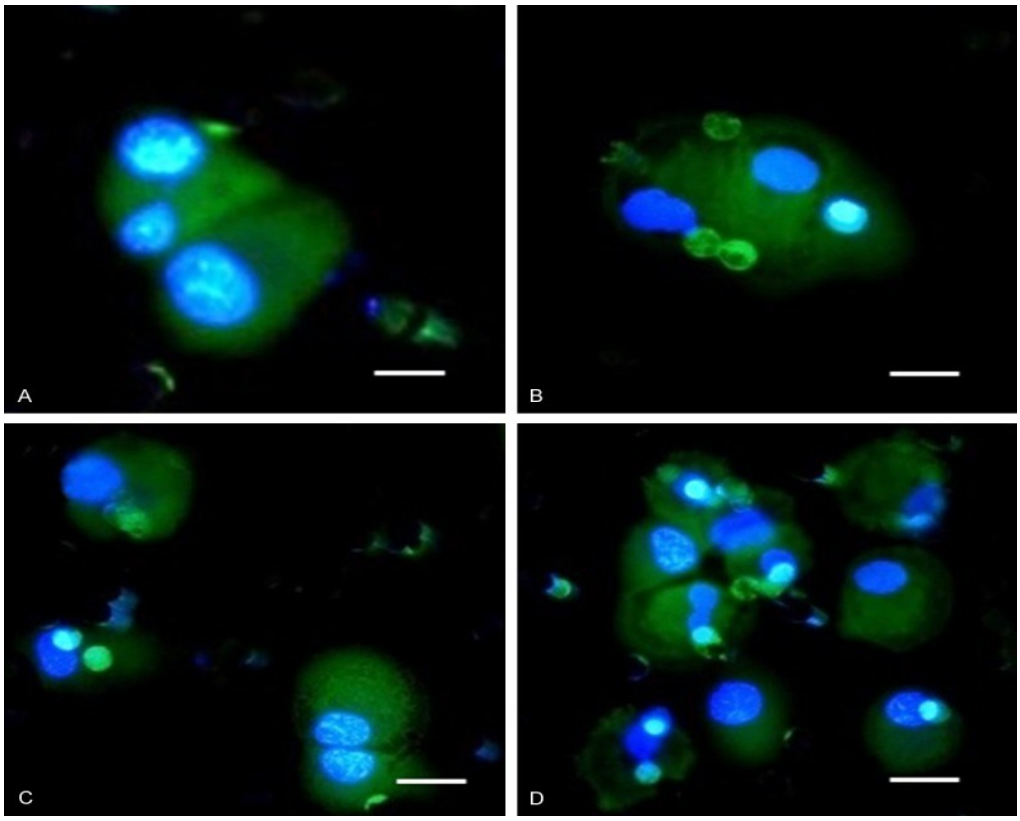

Figure 21: CTC of CaOV patient on the membrane after enrichment and cultivation in publication V. Cells were stained by NucBlue™ (nucleus, blue colour) and CellTracker™ (cytoplasm, green colour) and displayed by fluorescent microscope (pictures A-D). The bar symbolizes 10µm. CaOV, ovarian cancer (adapted from Kolostova et al., 2015)

Information on real time disease development in cancer patients can be obtained not only from CTC presence but also from their characteristics. One of the mechanisms of shedding the CTC from tumor tissue is EMT. Subpopulations of CTC with various morphological features and molecular character have been found. Moreover, CTC have been circulating in early tumor patient, too. (Chang et al., 2019; Guo et al., 2018; Zhang et al., 2018)

After cytomorphological analysis, genes expression analysis was performed in samples of 40 CaOV patients in **Publication IV** (page 261). Four fractions for every patient have been tested: peripheral blood leukocytes (fraction 1 sample type 1), CTC fraction stored immediately after enrichment procedure (fraction 2 sample type 2), cultured CTC fraction grown on the membrane (fraction 3 sample type 3)

and CTC fraction which pass through the membrane and grew at the bottom of cultivation plate (fraction 4 sample type 4). Relative gene expression for monitored markers regarding different sample types is shown on the figures below. (Figure 22 and Figure 23)

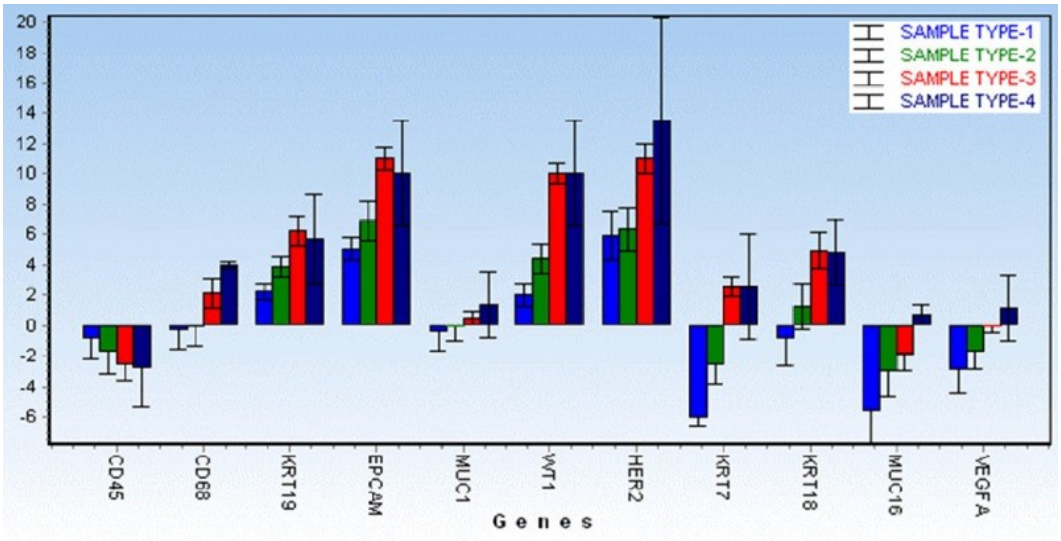

Figure 22: Relative gene expression of monitored markers regarding each sample types in publication IV. Sample type-1, whole peripheral blood; sample type-2, CTC fraction stored immediately after enrichment procedure; sample type-3, cultured CTC fraction grown on the membrane; sample type-4, CTC fraction which pass through the membrane and grew at the bottom of cultivation plate. (adapted from Kolostova et al., 2016)

The results of gene expression analyses showed noticeable increase of tumor-associated genes in both CTC - enriched and also CTC - cultivated fractions. Single CTC enrichment by size-based MetaCell® device had resulted in higher level of cytokeratin-7 (KRT7), KRT18, KRT19, EpCAM, wilms tumor 1 (WT1) and mucin 16 (MUC16) in CTC fraction if compared to peripheral blood samples (sample type 1). (Figure 22) Results had become more powerful in the case of enriched CTC fraction after short-term *in vitro* cultivation. Gene expression analyses revealed elevated level of KRT7, KRT18, KRT19, EpCAM, MUC1 and MUC16 in fraction 3 in comparison with peripheral blood with statistically significant differences ($p < 0.02$). (Figure 22 and Figure 23) These results had strongly indicated not only presence of cancer cells in our samples but also revealed their detailed characteristis.

In the next step, gene expression analysis of chemoresistance-associated genes was performed. The analysis included following genes: MRP1, MRP2, MRP4, MRP5, MRP7, ATP binding cassette subfamily B member 1 (MDR1) and ERCC1. According to the findings, all of these genes were expressed in excess if compared to peripheral blood. (Figure 23)

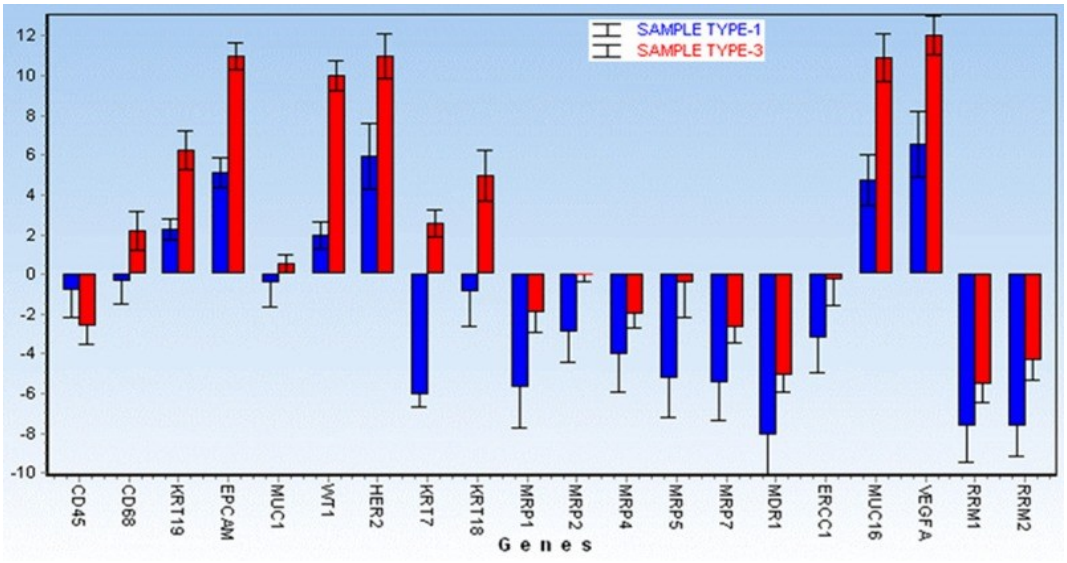

Figure 23: Relative gene expression of all of the monitored markers considering two sample types in publication - whole peripheral blood and cultured CTC fraction grown on the membrane. Sample type-1, whole peripheral blood; sample type-3, cultured CTC fraction grown on the membrane. (adapted from Kolostova et al., 2016)

Comments and conclusion of CaOV research are described below

This research has been focused on CTC examination by the means of CTC enrichment, cytomorphological and molecular analysis in patients with different stages of CaOV. Since this disease is known for its aggressiveness and usually later stages diagnosis, biomarkers for recognition and monitoring have been studied for decades. Although the progress in this field was remarkable and initial therapy (surgery, chemotherapy) is usually successful, recurrence and treatment failure occurs in most of the patients. New biomarkers are still essentially needed for better outcome of CaOV patients' achievement. (Markowska et al., 2018; El Bairi et al., 2017)

One of the most challenging parts within CTC analysis process is enrichment mainly because of their rarity and heterogeneity. (Ma et al., 2018) Enriched sub-population of cells should represent actual situation of cancer cells in peripheral

blood of the individual patient which is not the case of one or several specific markers-based isolation. (Muinao et al., 2018; Markowska et al., 2018) In our study size-based enrichment by MetaCell® was used and vital cytomorphological analysis revealed 58% and 65.2% as CTC-positive in **Publication IV** (page 261) and **Publication V** (page 269), respectively. These results were in agreement with gene expression analysis in 92% of samples. Based on the results, we can conclude size-based enrichment by MetaCell® is appropriate method for viable CTC enrichment in CaOV. CTC are able to grow and divide under the laboratory conditions for both short- and long-term (not a part of presented CaOV publications) cultivation.

Significant correlation had been confirmed between CTC presence and ascites, peritoneal carcinomatosis and residual disease. Our results had out performed the hypothesis that CA125 levels and CTC are two independent CaOV markers, which has been confirmed in other studies, too (Cheng et al., 2017)

Microscopic analysis was followed by multimarker genes expression examination. Since CTC population is not homogenous, tumor- and chemoresistance-associated markers were tested for deeper revealing of CTC character in our samples. Our heterogeneous genes expression results suggest our enrichment method is effective enough to obtain representative CTC patients sample. Increased expression not only tumor-associated but also chemoresistance-associated genes in our patients' samples were detected. Features of CTC have been also developing through time based on current environment (f.e. chemotherapy) which can result from therapy failure. (Chebouti et al., 2017) Moreover, CTC may be useful biomarker of minimal residual disease detection after surgery removal of tumor in CaOV patients. (Obermayr et al., 2017) Real-time monitoring can be helpful in early detection and therapy response prediction. (Zhang et al., 2018; Cheng et al., 2017)

4.3. Other cancer diagnosis

Patients diagnosed with another cancer(s) were monitored during their treatment process in the form of individual case studies. The **Publication III** (page 244) has been issued as a report of 8 cancer patients with CTC dynamics long-term monitoring in time. Patients were diagnosed with BC (case studies of patients' no.

1-4 are presented under “Breast cancer” section of Results and comments), CRC, prostate and lung cancer.

Following questions were asked regarding CTC study in various cancer diagnoses

The study should answer the question, how to use CTC in clinical practice based on the data with obtained in time following the dynamics of the cancer disease.

Do CTC reflect whole cancer disease heterogeneity?

Are CTC ready to be used in clinical practice at all?

Could be a personalized therapy implemented at all?

Results of research are described below

Publication III (page 244)

Patient No. 5 (CRC; 35 years old, man) underwent CTC examination after primary surgery and two relapses. This withdrawal was evaluated as CTC positive where KRT18, KRT19, MUC1 and MRP4 genes showed increased expression. After FOLFIRI (leucovorin + fluorouracil + irinotecan) chemotherapy regimen and after the third relapse, another CTC analysis revealed higher expression of KRT18, KRT19, MUC1, VIM, Ribonucleotide Reductase Catalytic Subunit M1 (RRM1) and MRP1 genes. RAS status was examined with negative results for liver metastasis and CTC.

Patient No. 6 (Rectal adenocarcinoma, 50 years old, man) started with CTC examination after surgery and lungs generalization. All of the CTC analyses were evaluated as CTC positive. The cells expressed epithelial characteristics, chemoresistance profile showed MRP1 and ERCC1 in the first blood withdrawal and two additional genes (RRM1, Ribonucleotide Reductase Regulatory Subunit M2 (RRM2)) were elevated in the second withdrawal. The third CTC analysis was provided by means of cytomorphology only, the last CTC test revealed increased expression of KRT18, thyroid transcription factor 1 (TTF1), MRP1 and MRP4 genes in CTC fraction. The disease progressed and new lesions in bones were detected. This patient died because of new lesions in CNS.

Patient No. 7 (Lung cancer, 47 years old, man) who's first CTC analysis was provided during carboplatin and pemetrexed therapy resulted in detection of elevated gene expression for: KRT18, KRT19, EpCAM, MUC1, MRP1 and ERCC1. Later on, CTC examination revealed not only mesenchymal characteristics, VIM expression, but also more chemoresistance-associated markers, such as MRP1, MRP2, MRP4, MRP7 and ERCC1.

Patient No. 8 (Duplicate tumor CRC and PC; 57 years old, man) monitoring CTC analysis revealed there are cells expression epithelial (KRT18, KRT19), stem cells-like (VIM, ALDH), prostate (AMACR) and vascular endothelial growth factor (VEGF) markers in the patient peripheral blood.

Comments and conclusion of research are described below

The **Publication III** (page 244) reports long-term monitoring of cancer patients, for whom the CTC examination might bring unique treatment management option. Their status in aspects of CTC-count and CTC-character has been changing dynamically through treatment process.

Another step in presented CTC examination is gene expression analysis. Results of our study have supported the opinion of CTC dynamic changes during therapy. Changes of chemoresistance-associated markers expression have occurred frequently. Increasing levels of these markers can also reduce therapy efficiency (patient no. 1, 6). These dynamics of CTC can allow real-time monitoring of individual patients disease status.

The heterogeneous nature of tumor disease has been confirmed in various cancer types. Researchers have revealed this character at the multiple levels; between primary tumor and metastasis, between metastasis themselves and also within tumor tissue. (Sokolenko and Imyanitov, 2018; Zhang et al., 2016) Moreover, primary tumor and CTC have not always shared the same characteristic. (De Kruijff et al., 2018; Liu et al., 2017; De Gregorio et al., 2017; Kulemann et al., 2017)

This publication had demonstrated individual development of cancer disease for each patient in various diagnoses and also supported idea of personalized therapy.

5. Discussion

Cancer is a systemic disease and its initial prognosis and predictions are not always consistent with the current disease development. Patients can experience increase in tumor volume or metastasis forming despite complete surgical removal of the primary tumor, receiving targeted medicinal products or general improvements in medical care.

For sure, a regular monitoring of the disease status is a necessary part of the treatment schedule and tumor biopsies and imaging methods are considerably involved in. These procedures have often an invasive character, especially in cases where tumor is not easily accessible due to its location. (Schaffner et al., 2020; Liikanen et al., 2018; Dasgupta et al., 2017; Tang et al., 2017; Wu et al.,2017; Zhang et al., 2017; Shiozawa et al., 2015; Tu et al., 2014; Pantel and Alix-Panabieres, 2014)

Cancer cells released from primary or metastatic tumor mass reaching patients'blood seem to be a useful marker for real-time tumor disease monitoring. However, their occurrence is very rare and their fate in circulation rather unclear. Although it has been suggested that CTC half-life in bloodstream is about 1-2 hours, these cells can be detected 8-22 years after mastectomy in BC patients. (Cote and Datar, 2016)

In fact, CTC examination relies on peripheral blood withdrawal which is a minimally invasive procedure. Peripheral blood, seen as a source of CTC for LB analysis, is the only prerequisite of the regular CTC-monitoring during whole treatment process. (Yamada et al., 2019; Zapatero et al., 2017; Potdar and Lotey, 2015; Karachaliou, et al., 2015) There are usually several treatment options considered for a patient before the final decision of the therapeutic schedule is agreed. Biomarkers are significant help within this decision processes. (Coates et al., 2015; Burstein et al., 2014) A biomarker has been defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention. (Kessler et al., 2015)

Enrichment of viable CTC is believed to play a significant role in experimental functional assays. CTC present a great challenge due to their natural characteristics such as incidence, heterogeneity or adaptability. Occurrence of CTC in patients' blood has been estimated to be 1 - 10 CTC in 10^6-10^8 WBC or 1-100 CTC may be found in 1 ml of whole blood. (Ecke and Thomas, 2019; Gard et al., 2018; Liu and Hu, 2017; Shen et al., 2017) Also, CTC are highly heterogeneous population considering both biological and physical properties. Enrichment strategies relying on a single CTC-feature has not obtained reproducibility and clinical relevance so far. A significant ratio of CTC is able to survive in unfavourable environment in circulation by several mechanisms such as phenotype alteration which avoids them from immune system attack. (Luo et al, 2018; Yang et al., 2017; Meyer et al., 2016)

Numerous methods have been developed for CTC enrichment and detection in the last decade. A size-based enrichment protocol (Metacell®) has been implemented in clinical studies of various cancer types. This technology allows obtaining of viable and unmodified CTC without implementation of pre-processing procedures or any physical force during enrichment. The artificial overpressure or vacuum which are used in the most of the size-based enrichment methods to regulate the filtration flow, can result in cells damage, too. (Bhagwat et al., 2018; Kang et al., 2017; Song et al., 2017; Gabriel et al., 2016) The size-based captured cells are heterogeneous, vivacious and contain both single cells and clusters that enable further downstream cellular and molecular analysis including set up of both short- and long-term *in vitro* cultures as has been discussed in presented **publications I-V**.

Research of CaOV CTC (**Publication V,** page 269) evaluated 118 patients with CaOV who were candidates for surgery or surgical diagnostics. 77 out of 118 (65.2%) patients were evaluated as CTC positive and short-term *in vitro* CTC cultures were established. Gene expression analysis confirmed heterogeneous expression profile of captured cells. The results confirmed that CA125 level and CTC may behave as two independent biomarkers for lymphogenic and haematogenous dissemination in CaOV patients, respectively.

In the study of Rao et al., CTC positivity was detected in 87% of patients with CaOV if CTC were captured by microfluidic system with immunomagnetic beads. (Rao et al., 2017) Other results have been reported by Obermayr et al. who observed CTC positivity at the time of diagnosis and 6 months after completion of adjuvant therapy in 26.5% and 7.7% of CaOV patients, respectively. CTC enrichment was provided by density gradient centrifugation and followed by multi-marker immunostaining. (Obermayr et al., 2017) Gebauer et al. reported CTC positivity in 32.3% of CaOV patients using CellSearch system and confirmed presence of CTC was not correlated with FIGO stage, nodal status or grading factors in CaOV patients. (Gebauer et al., 2017)

In the molecular study of CTC in patients with CaOV (**Publication IV,** page 261) cytomorphological and molecular analysis of enriched cells was performed. 56 patients with CaOV were enrolled into this study and 32 out of 56 (58%) were evaluated as CTC positive. Gene-expression analysis confirmed heterogeneity of the population of CTC enriched and cultured by size-based Metacell® protocol examinating tumor-associated genes expression. We found increased expression of EpCAM, KRT7, KRT18, MUC16 and WT1 genes in CTC-enriched fraction and elevation in EpCAM, KRT7, KRT18, KRT19, MUC1, MUC16 and WT1 genes expression after 3 days of CTC - *in vitro* culture. This study has also supported the idea of multi-marker instead of single-marker analysis. In the recent paper of Balakrishnan et al. enriched CTC were used to set up a short-term cluster culture. This study had confirmed that captured cells were cytokeratines positive and CD45-negative after 14 days of *in vitro* culture. (Balakrishnan et al., 2019) Outcomes of these studies support the hypothesis of immune cells depletion during *in vitro* culture.

Similar analysis was also performed in the study of 167 patients with various BC stages (**Publication II,** page 238). CTC examination revealed CTC positivity in 127 patients. Further cytomorphological and tumor-associated gene expression analysis revealed the highest CTC occurrence was identified in the group undergoing surgery and before the start of neoadjuvant treatment. In this study HER2 and ESR receptors, routinely examined in primary tumor, were evaluated in CTC. These markers are being used to stratify BC patients with early disease and are used as

predictive markers for targeted therapies, too. (Schaffner et al., 2020) It has been widely accepted that expression status of these key receptors differs between primary tumor and metastasis. (Aaltonen et al., 2017) **Our testing also found that status of these markers can differ between primary tumor and CTC.** Research in this area is still not delivering consistent results. Although some of the studies demonstrated significant discordances in receptors status on CTC and in primary tumor tissue, other studies reported similarities between them. (Braun et al., 2019; Wang et al., 2017; Beije et al., 2016; Wallwiener et al., 2015; Kalinsky et al., 2015; Onstenk et al., 2015) One of the possible explanations could be coexistence of cells that display different phenotypic characteristics. (Castro-Giner and Aceto, 2020; Jordan et al., 2016) In any case, this discordance can significantly affect treatment response of BC patients.

Long-term CTC monitoring was performed in the study presented as collection of case reports (**Publication III,** page 244) where patients with BC, CRC, prostate and lung cancer were involved. Individual cases were analysed for CTC and clinical perspective of the CTC test results. In this publication, several aspects of CTC monitoring are described. Cytomorhological evaluation was concentrated on showing CTC numbers in responders and non-responders. The CTC numbers differed in the patients with the same disease type undergoing the same treatment. Size-based label-free enrichment was used and heterogeneous CTC population was obtained. In total, 24 out of 34 (70.6%) samples were evaluated as CTC-positive. Molecular analysis revealed that EpCAM, KRT18/19 and MUC1 gene expression was increased in 2, 23 and 7 out of 34 samples, respectively. This study supports the idea of the long-term cancer patients monitoring. Continuous CTC-monitoring after primary treatment is a tool how to follow the CTC-persistence in the blood. Persistence of CTC after primary treatment significantly increases relapse risk. Recent study of Trapp et al. published that CTC presence 2 years after chemotherapy significantly increased a risk of relapse in eBC patients. (Trapp et al., 2019) Similar outcomes have been reported by Sparano et al. who studied association of CTC with late recurrence of ESR+ BC patients. They reported that CTC positivity 5 years after diagnosis had provided independent prognostic information for clinical recurrence. (Sparano et al., 2018)

The last presented study of eBC patients (**Publication I**, page 212) included a long-term monitoring for both cytopathological and molecular CTC character of 20 patients diagnosed with eBC. These patients underwent CTC examination in the time of diagnosis, during NACT (after AC therapy, after TAX treatment), after surgery and in the next follow-up period. We monitored CTC during the course of treatment and found that CTC-positivity is not associated with tumor volume. The study confirmed that receptors status is not always the same in primary tumor and CTC. According to our results, during NACT, the highest concordancy rate in receptor expression was observed in patients with TNBC and HER2+ primary tumors. On the other hand, CTC of patients with ESR+ primary tumors showed the lowest concordancy rate regardless of their HER2 status. Long-term monitoring revealed each of the patients monitored experienced an individual disease development. Our publications emphasize importance of personalized treatment.

Although chemotherapy regimens are often an essential part of the cancer treatment, its success rate can be low due to intrinsic or acquired chemoresistance mechanisms. (Briz et al., 2019) Monitoring of genes associated with chemoresistance has become an important part of our CTC studies. We monitored gene-expression of chemoresistance-associated genes in different cancer case studies and in the study of early BC patients (**Publications II and III**, pages 238 and 244). In the **Publication III** (page 244) a prolonged CTC-monitoring revealed an expression of different chemoresistance-associated genes for various diagnoses. The development of chemoresistance has been confirmed also in eBC patients (**Publication I**, page 212**).** Despite importance of individual features of the disease progress, some of the findings were significant and common for several patients. In non-responders group, two or more chemoresistance-associated genes often showed increased expression and these CTC characteristics changed dynamically during treatment process. Worse therapy outcome was observed in patients with CTC that expressed chemoresistance markers. Frequent overexpression of MRP1, MRP2 or MRP7 was observed in our patients although another 9 patients with TAX chemoresistance showed elevated expression of MRP2, MRP7 or MDR1 before or during TAX therapy. Based on our observations, resistance patients expressed elevated levels of MRP1 during AC and MRP1 and MRP7 during TAX treatment.

ERCC1 overexpression was detected in 4, 3 and 2 patients before, during and after NACT, respectively and MRP1 was co-expressed in 8 of the 9 cases. We have also observed epithelial signs of CTC and minimal chemoresistance-associated genes expression during NACT correlated with positive clinical effect of AC or TAX.

Recent research of Shlyakhtunou et al. confirmed CTC heterogeneity in primary non-mBC patients. This study has also revealed elevated expression of MRP7, MRP1, MDR1 and MRP5 in 37/69 (54%), 37/69 (54%), 24/69 (35%) and 14/69 (21%) of BC patients included in this study, respectively. (Shlyakhtunou, 2018) Gradilone et al. classified patients into "drug sensitive" and "drug resistant" groups based on MRPs expression and found that the resistant group has significantly shorter PFS than the sensitive patients. Detailed analysis also confirmed that patients whose CTC expressed two or more MPRs were having shorter PFS than those who expressed zero or one of the MRPs. Furthermore, this research had clearly connected MRP1 and MRP7 with resistance to AC and TAX, respectively, which is in concordance with our results. (Gradilone et al., 2010) In another study Kasimir-Bauer et al analysed ERCC1 in CTC. 72% of residual CTC obtained from peripheral blood of BC patients after NACT expressed ERCC1 which indicated them as therapy resistant population. Awareness of this information may help clinicians with decision of further therapy. (Kasimir-Bauer et al., 2016) Approaches which would be able to pair treatment targeting the predominant, drug-sensitive population in addition to various subsets of drug-resistant and drug-tolerant cells may lead to the most-durable responses achievement. (Dagogo-Jack and Shaw, 2017)

EMT and stem cell-like characteristics of cancer cells have been also considered as major importance in metastatic progression. (Schaffner et al., 2020) Previous studies confirmed presence of tumor cells which survive in the blood, pass through EMT and can exist in intermediate stages expressing both epithelial and mesenchymal markers. This cell phenotype has been found to contribute to resistance of anti-cancer therapies including AC and TAX. Part of this heterogeneous CTC population may manifest stem cells character. The stem cell character promotes therapy resistance, survival facilitation or metastases. (McInnes

et al., 2015; Yu et al.,2013; Bonnomet et al.,2012; Lecharpentier et al., 2011; Raimondi et al., 2011) Although CTC with elevated expression level of EMT or intermediate phenotype occured more frequently in patients with metastatic in comparison with early-stage disease, their presence is not rare so their significance should not be underestimated. (Lowes and Allan, 2018; Pal et al., 2015; Papadaki et al., 2014)

Markers that characterize these features were included into our testing. We have identified these types of cells by CD24, CD44 in study of eBC patients (**Publication I**, page 212**)** and together with ALDH1 and VIM genes-expression analysis in study of case reports (**Publication III**, page 244**).** We observed differences in stem cells markers expression in ESR+ and other BC subtypes in study of eBC patients (**Publication I**, page 212**)**. Although in the cases of ESR+ primary tumor CTC with CD24/CD44 characteristics were detected more often after surgery in comparison with epithelial features (patient no. 1, 2, 7, 14 or 15), patients with more aggressive tumor types (HER2+ primary tumor diagnosis) showed CTC with these markers overexpressed from the beginning of disease (patient no 9, or 12). However in study of eBC patients (**Publication I**, page 212**)** most of the TNBC patients overexpressed CD24 and/or CD44 markers in their CTC from the time of diagnosis (patient no. 10, 11, 13, 16, 17, 19, 20), study of case reports (**Publication III**, page 244**)** showed 3 TNBC case reports each with different EMT and stem cell-like features during CTC development. In the patient no.1 we observed changes in CTC regarding EMT and stem cells-like characteristics. None of these markers was overexpressed in the CTC from the first blood withdrawal, VIM, ALDH1 and CD44 gene expression was elevated in the second, third and fourth blood collection, respectively. The expression level of CD24 and CD44 elevated from the diagnosis time point and during the most of her blood withdrawals in the CTC of patient no. 2. As next, VIM and ALDH1 markers were overexpressed later during the monitoring, after 15 months. Recent study of Akkiprik et al. reported CTC with stem-cells properties were found in most HR+, HER2- cases and their high incidence was also observed in the cases of early metastasis. (Akkiprik et al., 2020)

Our studies declare patients individual CTC development in time considering the different markers monitored. Most of the BC patients included in our studies

overexpressed EMT and stem cells-like markers in the corresponding CTC. Presented studies have also contributed to the hypothesis that circulating cancer cells may become a helpful tool for individual patients‘disease characterization and provide benefit for early detection and therapy personalization as transition status between epithelial and mesenchymal cells subpopulation changed dynamically. (Mansoori et al., 2017; Grosse-Wilde et al., 2015; Liu et al., 2014)

Despite all efforts, metastasis may take place after a long period after surgical tumor removal without any symptoms' appearance. Metastasis has been major cause of cancer-related death. Understanding and proper monitoring of this process may bring disease treatment process improvement. (Chitty et al., 2018; Gomis and Gawrzak, 2017; Liu et al., 2017)

CTC showed to reflect cancer disease more accurately than biopsies, they are highly heterogeneous and more affortable in comparison with classical biopsies. Additional studies are needed to explore their use and potential in cancer disease monitoring, treatment and patients' life quality improvement. (Brown et al., 2019; Ye et al., 2019; Jie et al., 2017; Aaltonen et al., 2017; Gkountela et al., 2016)

6. Conclusion

The presented PhD thesis was focused on CTC, their isolation, *in vitro* culture, cytomorphological description and molecular characterization. Based on our results, following could be concluded:

1. Size-based enrichment (MetaCell®) of CTC in patients diagnosed with solid tumor diagnoses was performed and successfully implemented into the diagnostic processes, reflecting purposes of special timing for CTC-tests in different cancer treatment periods (neoadjuvancy, adjuvancy, palliative). Patients with BC, CaOV, lung, CRC and prostate cancer were included into the testing. The enriched CTC (bigger than 8μm) were captured in a viable stage and were in the good fitness cultured for both, short and long time period *in vitro.*
2. Enriched and cultivated cells were observed using vital fluorescent microscopy. Following cytomorphological criteria were applied and evaluated to identify tumor cells in CTC samples: nuclei larger than 24μm, cell size at least 1.5 times larger than white blood cells, irregular nuclei and nuclear membrane, anisonucleosis, high nuclear/cytoplasmic ratio, presence of 3-dimensional cell sheets, presence of prominent nucleolus or several nucleoli. Significant differencies in CTC morphology of BC patients during treatment included also prevalent changes in CTC number and increased frequency of CTC clusters formation.
3. Gene expression profiling of both tumor- and chemoresistance-associated genes was provided in CTC collected during NACT, surgery and in follow-up period in BC patients. CTC expression profile has been analyzed reflecting disease and treatment dynamics. In patients who did not respond to NACT, two or more chemoresistance-associated genes showed elevated expression. The molecular characteristics of CTC were changing dynamically during treatment process.
4. It was confirmed that molecular characteristics of tumor and CTC in BC patients may not be always concordant either in the time of diagnosis or

during treatment period. This has been confirmed in every of the presented publications (I-V). In the **Publication III** (page 244), CTC of TNBC patients have been HR and/ or HER2 negative in 78.5% of tested cases. In the **Publication V** (page 269), the change from HER2 negative to HER2 positive status and *vice versa* was shown for 33.3% and 50% of BC patients, respectively. Status of HR (ESR) changed from ESR+ to ESR- in all of the patients monitored (3/3). Based on the results from **Publication IV** (page 261) a high concordance rate of CTC and primary tumor was confirmed for TNBC and HER2+ BC patients. The highest discordance was revealed in tumors with ESR+ status independent of HER2 status.

We can conclude that majority of CTC showed ESR- and HER2-negativity in HER2-negative primary tumors. On the other hand, in HER2-positive primary tumors, discordance of HER2 was detected in 50% of tested CTC samples.

5. Gene expression analysis of CTC identified several CTC subpopulations in BC patients' samples. We were able to identify CTC with epithelial, mesenchymal, stem cell-like and chemoresistance features during long-term monitoring of CTC (up to 36 months in total).
6. Long-term monitoring in combination with real-time clinical assessment (ultrasonography) had allowed us to conclude that CTC of NACT responders overexpressed epithelial genes. In the case of the NACT non-responders, increased expression of chemoresistance-associated genes in CTC of these patients was detected.

7. Prospective studies

The intensive research of CTC has been conducted for the last sixteen years despite the fact CTC were first described in 1869 by Thomas Ashworth. The milestone for the increased interest was the work of Cristofanilli et al. that suggested CTC as potential prediction marker of OS and PFS. (Cristofanilli et al., 2004) Although during this period significant progress has been done in CTC characterization, more studies and investigation are necessary to reach and use full potential of CTC.

In future, two main challenges of CTC need to be overcome: rarity and heterogeneity. These features cause their problematic enrichment and raise question regarding ability to obtain representative CTC sample from cancer patient. Numerous techniques based on different principles have been developed for CTC enrichment and detection; new approaches are still currently tested. Time necessary for processing or procedure types may cause damage to CTC and accurate CTC identification has remained challenge for researchers. (Yang et al., 2021; Cote and Datar, 2016)

The other approach that could help with CTC enrichment is to increase the volume of the tested blood. The preliminary results suggested large volumes of blood from patient could significantly improve CTC enrichment efficiacy. It has been found that detection rate difference in 7.5 and 30 ml of blood sample is 13% and 47%, respectively. Practical outcome for the patients in the future could be either large sample volume or multiple blood withdrawals. (Eifler et al., 2011, Lalmahomed et al., 2010) Another question is setting up the correct CTC withdrawals frequency. Whereas low frequency could cause incorrect assumption or delayed diagnosis, too high frequency can result from misleading trend due to CTC oscilation. Number of studies need to be performed to set up the correct blood withdrawal frequency. (Leong et al., 2015) The fourth approach how to obtain representative CTC sample is to stabilize blood where CTC are enriched from. The fitness and vitality of the cancer cells in the collected blood is an important factor for their enrichment, characterisation and downstream analysis. (Yang et al., 2021)

The current effort is to use information obtained from CTC to help with cancer management and patients' treatment process. Techniques of molecular analysis could be applied in personalized therapy and may help with cancer patients' assignment into high- or low- risk group. Another approach could be applied in therapy regimens selection. Due to the fact that CTC are living and also sometimes proliferating, their characterisation could make us an advantage to be one step ahead – ability to identify prominent genetic feature in the disease relapse. (Diamantopoulou et al., 2020, Chemi et al., 2019)

Future approach for treatment decision could also include *ex vivo* culture and drug screening. In the case of cancer patients therapy failure events are not rare particularly in advanced stages of disease when multiple therapeutics failures were observed. In these cases, small-scale model of the patients' disease could help with identification of effective treatment combination. The limitation of this approach is time as cells need to be cultivated and following testing will take place in weeks or months period after blood withdrawal that can not reflect the current situation. (Diamantopoulou et al., 2020)

The current idea of future CTC management is to obtain real-time information about disease status in individual patients. Potential of using cancer cells from blood is to identify aggressive CTC, new targets for therapies, discover cellular markers and prevent of metastatic disease.(Li et al., 2020; Maheswaran and Haber, 2010)

8. Summary

Circulating tumor cells (CTC) have showed great potential to become both prognostic and predictive biomarker in various types of oncological diseases. CTC can help detect patients in higher risk of shorter overall survival, progression-free survival or relapse. They can be also helpful in therapy selection as in current clinical practice treatment is chosen based on primary tumor characteristics. Regular CTC counts and features monitoring can be real-time indication of therapy response and can be used to guide-targeted treatment. This information can be implemented to personalized medicine and each cancer patient can be treated based on individual profile.

However, patients' sample with CTC is easily accessible, their detection has remained challenge due to low CTC number in the circulation and heterogeneous nature. CTC can circulate in blood in the form of single cells or in clusters that usually represent minority in comparison with single CTC but their metastatic potential is significantly greater than of single CTC. Apart from CTC count, the molecular character showed dynamic development and heterogeneous nature not only between patients but also within the individual patient's tumor tissue itself. Character of primary tumor, CTC and metastasis are not always consistent and has been changing during treatment process which can significantly impact response to therapy.

In this work, we used size-based CTC enrichment in various cancer diagnoses and monitored both CTC count and molecular character in regular intervals during treatment process. Cytomorphological and genes-expression analyses revealed dynamic disease development through whole treatment process. Fluorescent microscopy found count changes not only single CTC but also in CTC clusters presence. CTC behaviour also varied during withdrawals. While some of the CTC were able to survive only days during cultivation, there were enriched CTC fractions with aggressive growth and long-term cultures were established from them. Gene-expression CTC analyses of genes associated with tumor, epithelial-mesenchymal transition, stem cells-like features and chemoresistence revealed their presence and dynamic change in expression levels. We found CTC character does

not correlated to corresponding primary tumor features. The outcomes of the CTC research are summarized in publications that are part of this doctoral thesis.

9. Souhrn

Cirkulující nádorové buňky (CTC) prokázaly velký potenciál stát se prognostickým a prediktivním biomarkerem u různých typů onkologických onemocnění. CTC by mohly pomoci detekovat pacienty s vyšším rizikem kratšího celkového přežití, přežití bez progrese nebo relapsu. Mohou být také užitečné při výběru terapie, protože v současné klinické praxi je léčba volena na základě charakteristik primárního nádoru. Pravidelné sledování počtu a charakteru CTC by mohlo být indikátorem terapeutické odpovědi v reálném čase a bylo by možné jej použít k cílené léčbě. Tyto informace by bylo možné implementovat do personalizované medicíny a každého pacienta s rakovinou léčit na základě individuálního profilu.

Ačkoli vzorek pacientů s CTC je snadno dostupný, jejich detekce CTC zůstává výzvou zejména kvůli jejich nízkému počtu v krevním oběhu a jejich heterogenní povaze. CTC mohou cirkulovat v krvi ve formě samostatných buněk nebo ve shlucích, které se obvykle nevyskytují tak často jako jednotlivé CTC, ale jejich metastatický potenciál je významně větší než u samostatných CTC. Kromě počtu CTC, taky molekulární charakter vykazoval dynamický vývoj a heterogenní povahu nejen mezi pacienty, ale také v rámci nádorové tkáně pacienta. Charakter primárního nádoru, CTC a metastáz není vždy konzistentní a během procesu léčby se mění, což může významně ovlivnit reakci na léčbu.

V této práci jsme použili metodu izolace CTC založenou na jejich velikosti u různých nádorových diagnóz a sledovali jsme počet a molekulární charakter CTC v pravidelných intervalech v průběhu léčby. Analýza cytomorfologie a genové exprese odhalila dynamický vývoj onemocnění v průběhu celého procesu léčby. Pomocí fluorescenční mikroskopie jsme pozorovali změny počtu nejen jednotlivých CTC, ale také CTC shluků. Vlastnosti CTC se také měnily mezi jednotlivými odběry krve. Zatímco některé CTC dokázaly během kultivace přežít jen několik dní, u jiných izolovaných CTC byl pozorován agresivní růst a založili jsme z nich dlouhodobé kultury. Analýzy genové exprese genů spojených s nádorem, epiteliálně-mezenchymálním přechodem, vlastnostmi podobnými kmenovým buňkám a chemorezistencí u CTC odhalily jejich přítomnost a dynamické změny v expresi markerů. Zjistili jsme, že charakter CTC nekoreluje

s charakteristikami odpovídajícího primárního nádoru. Výsledky výzkumu CTC jsou shrnuty v publikacích, které jsou součástí této disertační práce.

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11. List of tables

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13. Publications

I. **JAKABOVA Anna,** Zuzana BIELCIKOVA, Eliska POSPISILOVA, Lubos PETRUZELKA, Piotr BLASAK, Vladimir BOBEK and Katarina KOLOSTOVA. Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy. *Therapeutic Advances in Medical Oncology* [Accepted on 09 June 2021, Impact Factor in 2021: 7.03]

II. **JAKABOVA, Anna**, Zuzana BIELCIKOVA, Eliska POSPISILOVA, Rafal MATKOWSKI, Bartlomiej SZYNGLAREWICZ, Urszula STASZEK-SZEWCZYK, Milada ZEMANOVA, Lubos PETRUZELKA, Petra ELIASOVA, Katarina KOLOSTOVA and Vladimir BOBEK. Molecular characterization and heterogeneity of circulating tumor cells in breast cancer. *Breast Cancer Research and Treatment*. 2017, **166**(3), 695-700. ISSN 0167-6806. doi:10.1007/s10549-017-4452-9 [Impact Factor in 2017: 3.855]

III. BIELCIKOVA, Zuzana, **Anna JAKABOVA**, Michael PINKAS, Milada ZEMANOVA, Katarina KOLOSTOVA and Vladimir BOBEK. Circulating tumor cells:: what we know, what do we want to know about them and are they ready to be used in clinics? *American Journal of Translational Research*. 2017, **9**(6), 2807-2823. [Impact Factor in 2017: 3.255]

IV. KOLOSTOVA, Katarina, Michael PINKAS, **Anna JAKABOVA**, Eliska POSPISILOVA, Pavla SVOBODOVA, Jan SPICKA, Martin CEGAN, Rafal MATKOWSKI and Vladimir BOBEK. Molecular characterization of circulating tumor cells in ovarian cancer. *American Journal of Cancer Research*. 2016, **6**(5), 973-980. [Impact Factor in 2016: 3.265]

V. KOLOSTOVA, Katarina, Rafal MATKOWSKI, Marcin JEDRYKA, Katarzyna SOTER, Martin CEGAN, Michael PINKAS, **Anna JAKABOVA**, Jiri PAVLASEK, Jan SPICKA and Vladimir BOBEK. The added value of circulating tumor cells examination in ovarian cancer staging. *American Journal of Cancer Research*. 2015, **5**(11), 3363-3375. [Impact Factor in 2015: 2.125]

13.1. Publication I

Article Title: Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy

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Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy

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Running title: Characterization of CTCs in early breast cancer

Key words: CTCs; circulating tumor cells; breast cancer; cultivation; *in vitro*, gene expression; neoadjuvant chemotherapy; liquid biopsy

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Characterization of circulating tumor cells in early breast cancer patients receiving neoadjuvant chemotherapy

Abstract

Aim/Background: The aim of this study was to characterize circulating tumor cells (CTCs) during neoadjuvant chemotherapy (NACT) in early locally advanced breast cancer (LABC) patients. Using ultrasound, tumor volume measurement was compared to the presence and the molecular nature of CTCs over multiple time intervals corresponding to treatment periods.

Methods: Twenty patients diagnosed with breast cancer (BC) of different histotypes were monitored during the NACT period and in the follow-up period (~5years). Peripheral blood for CTCs (n= 115) was taken prior to NACT, after 2-3 chemotherapy cycles, after the completion of NACT (before surgery) and at some time points during adjuvant therapy. CTCs were enriched using a size-based filtration method (MetaCell®) capturing viable cells, which enabled vital fluorescence microscopy. A set of tumor-associated (TA) genes and chemoresistance-associated (CA) genes was analyzed by qPCR in the enriched CTCs fractions.

Results: The analysis of tumor volume reduction after administration of anthracyclines (AC) and taxanes (TAX) during NACT showed that AC therapy was responsive in 60% (12/20) of tumors compared to TAX therapy which was responsive in 30% (6/20; n.s.). After NACT, CTCs were still present in 70.5% (12/17) of patients (responders vs non-responders, 61.5% vs.100%; n.s.).
In triple-negative BC (TNBC) patients (n=8), tumor volume reduction was observed in 75% cases. CTCs were significantly reduced in 42.9 % of all HER2-negative BC patients. In HER2+ tumors, CTC reduction was reported in 16.6% only. Relapses were also more prevalent in the HER2-positive patient group (28.5 vs. 66.6%).
During NACT, the presence of CTCs (3 tests for each patient) identified patients with relapses and indicated significantly shorter progression-free survival (PFS) rates (p=0.03). Differentiation between progressive disease and non-progressive disease was obtained when the occurrence of excessive expression for CA genes in CTCs was compared (p=0.024). Tumor volume reduction was also significantly indicative for progressive disease (p = 0. 0224).
Disseminated CTCs in HER2-negative tumors expressed HER2 in 29% of samples collected during the overall follow-up period (16/55), and in 32% of samples during the follow-up of NACT (10/31). The change accounted for 78.5% of HER2-negative patients (11/14) in total. 63.6% of the conversion cases occurred during

NACT (7/11). For the remaining 4 patients (36.3%), conversion to HER2+ CTCs occurred later during adjuvant therapy. We believe there is a possibility to prevent further progression by identifying less responsive tumors during NACT using CTC monitoring, which could be effectively used also during adjuvant therapy.

Introduction

Neoadjuvant chemotherapy (NACT) is mainly indicated for locally advanced breast cancer (LABC), which involves stage IIB (T2N1, T3N0) and stage III, including inflammatory breast cancer (IBC). NACT is implemented to reduce tumor volume to convert inoperable to operable tumors or to replace mastectomy with breast-conserving surgery (BCS). NSABP B-18 and EORTC 1092 trials reported that application of NACT was connected mainly to downstaging of the disease (1-3). However, subgroup analysis of both trials showed the trend towards better outcome in patients under 50 years of age (1). The same benefit of adjuvant chemotherapy was reported in young premenopausal women (4) and women under 50 years of age (5). Moreover, early indication of NACT and response to doxorubicin (A) and cyclophosphamide (C) regimen yielded better disease-free survival (DFS) and overall survival (OS) compared to patients undergoing adjuvant treatment with docetaxel (D) in the B27 study (6).

The current indication for NACT is based on tumor biology and is considered a treatment option in BC patients with a high risk of tumor dissemination and worse prognosis (7). The most important finding in neoadjuvant studies is the association between pathological complete response (pCR) and long-term clinical outcomes. pCR occurs mainly in patients with aggressive BC subtypes (8). Tumors showing high proliferation (Luminal B [HR+, HER2+], HER2+, TNBC) have higher rates of pCR compared to Luminal A (HR+, HER2-) (9). Post-treatment residual disease and Ki67 levels also seem to have prognostic significance (10,11). It is reported that particularly patients with extensive residual disease and Ki67 > 35% after NACT have significantly worse outcomes. The need for new biomarkers reflects the shortcomings of existing therapeutic options. Although AC and TAX regimens in NACT are considered standard clinical practice, recurrence due to clonal expansion and/or resistance of residual tumor cells due to treatment selection pressure occurs.

The metastatic potential of BC could be stratified not only by primary disease subtype, but also could be measured by the presence of CTCs. Although their occurrence in the blood is very rare, CTCs have a significant prognostic value in patients with primary BC (PBC) and metastatic BC (MBC) (21-23). However, the clinical utility based on the predictive value of CTC enumeration remains uncertain. The genomic characteristics of CTCs may indeed be more important for therapy recommendation as suggested by the SWOG S0500 trial (24) as well as enhancement of patient outcomes (25,26).

Clusters are one of the typical CTC characteristics that can be present in patients with BC. CTCs that are bound together may exhibit 23- to 50-fold higher metastatic potential than single CTCs (27,28). The presence of these clusters in peripheral blood of patients has been clearly associated with shorter progression-free survival (PFS), metastatic-free survival (MFS) and overall survival (OS) in various types of

BC compared to individual CTCs (28,29).

Many enrichment methods have been successfully implemented into laboratory practice to enrich, detect and isolate CTCs from a simple blood draw. Immunomagnetic and size-based isolation techniques are the most commonly used (30-33). CTCs can be detected by immunofluorescence staining (34), laser scanning cytometry (35) or quantitative polymerase chain reaction (qPCR) (36).

In the case of BC, several markers are known to have different expression ratios when non-malignant normal cells are compared to tumor cells, such as cytokeratin 19 (KRT19), human epidermal growth factor receptor 2 (HER2), epithelial cell adhesion molecule (EpCAM), mucin 1 (MUC1), epidermal growth factor receptor (EGFR), mammaglobin (MGB), and maspin.

Another promising marker that can be potentially used for BC diagnostics and therapy is the disialoganglioside GD2 subtype (GD2) due to its overexpression in tumors (37,38). A high prevalence of GD2 in aggressive BC subtypes such as MBC and TNBC was observed (39,40).

A new targeted therapy with glembatumumab vedotin (CDX-011) is currently under evaluation in clinical trials. Its aim is to attack the glycoprotein non-metastatic b (GPNMB) (41-43). Similarly, clinical trials confirmed the efficacy of sacituzumab govitecan-hziy, which is a trophoblast cell surface antigen 2 (TROP2)-directed antibody-drug conjugate. Other clinical trials in TNBC and other MBC forms are currently ongoing (42, 44).

Individually, these markers could be used for CTC identification at the molecular level. However, even greater specificity can be achieved by using a multi-marker assay to profile each marker simultaneously (45-48). In previous studies, the size-based filtration system (MetaCell®) was found to reliably recover viable CTCs from BC patients for cytomorphologic evaluation. It also permitted CTC downstream molecular characterization.

Despite clinical advances, in some patients, minimal residual disease (MRD) can persist and give rise to clonally advanced and resistant disease. Although it is known that phenotypic differences between primary tumor and CTCs exist (49-53), patients are still treated according to primary tumor characteristics. Recent therapeutic indications do not reflect the dynamic changes that occur in tumor cells, which are the target of chemotherapy. In this study, we compared tumor response during NACT with anthracyclines (AC) and/or taxanes (TAX) as measured by tumor volume with the presence and characterization of CTCs over multiple time intervals corresponding to standard treatment cycles. We reported the use of real-time molecular characterization of chemoresistance and tumor-related genes

following size-based enrichment (MetaCell®) of CTCs in predicting therapeutic decisions and PFS.

Patients and methods

Study design

In total, 20 BC patients undergoing NACT were enrolled in the study. The analysis was comprised of 115 blood samples obtained during regular medical examinations between 2014 and 2016. CTC assessment was performed prior to NACT, during NACT and/or before and after surgery. If NACT included sequential administration of AC and TAX, blood collection was usually performed before the first AC cycle, before the first TAX cycle and before the last TAX cycle. CTCs were enriched from peripheral blood (8 ml) by size-based filtration (MetaCell®, Czech Republic) (54). To provide more detailed study protocols, the figure is given to show a treatment plan for each patient in the Supplementary file SF1 Patient Follow-up. Figure 1 is an example and is related to patient no.7.

Patient characteristics

Table 1 and Supplementary File ST1 show patient characteristics. The median age of the group was 39 years. In the study, only one patient was postmenopausal (aged 71 at diagnosis). Of NACT-indicated patients, 16 subjects were treated for LABC with lymph node involvement. Negative lymph nodes (N0) were reported in 4 of 20 patients.

Based on histological evaluation, BC was classified as invasive carcinoma of no special type (NST) in 5 cases, invasive ductal carcinoma (IDC) in 14 cases, or medullary carcinoma in 1 case. Most cancers (16/20) were poorly differentiated (grade 3; G3), 3/20 carcinomas were graded G2 and 1/20 carcinoma was well differentiated (G1).

All patients presented with very aggressive disease based on proliferation parameters (Ki67) of tumor cells. 19/20 primary tumors exhibited Ki67 expression of at least 40%. Altogether, 12/20 tumors were estrogen receptor positive (ER+) and/or progesterone receptor positive (PR+), 6/20 patients were HER2 positive (HER2+). 9/20 patients were classified as TNBC. Patient no 7 was also classified as TNBC given that postoperative ER and PR status was negative. According to the subtype classification, 5 of 20 patients were HR+/HER2- (luminal A), 4 of 20 patients were HR+/HER2+ (luminal B, HER2+), 2 of 20 patients were HR-/HER2+ (HER2-amplified/overexpressed), and 9 of 20 patients were TNBC (ER-, PR- and HER2-).

Clinicopathologic features of each patient enrolled in the study (N=20) are reported in detail in Supplementary Table ST1, including details on individual risk (mutation, pregnancy etc.). NACT regimens and chemotherapeutic dosing are

reported in Supplementary file SF2 Methods.

CTC examination

CTCs were enriched from peripheral blood (EDTA/6-8 ml) by a size-based filtration method (MetaCell®, Czech Republic) (54). The enriched cells were incubated for 3-5 days *in vitro* (37ºC, 5% CO_2) and assessed in a two-step manner.

Cytomorphologic evaluation of the viable cells by vital fluorescence microscopy (NucBlue®, Celltracker®, Mitotracker®, ThermoFisherScientific, U.S.) was followed by qPCR analysis of RNA isolated from CTC fraction. qPCR analysis included tumor-associated (TA) and chemoresistance-associated (CA) genes. For more details, please see Supplementary file SF2 Methods.

Patient blood samples were classified as CTC-positive by combined microscopic evaluation and molecular analysis. In the cytomorphological analysis, fluorescently stained viable cells were scored according to the following criteria: nucleus size, nuclear membrane irregularity, prominent nucleoli, nucleoli count, cell size, and the presence of 2D and 3D cell sheets, etc. The recovered size-enriched fractions of cells captured on the membrane were lysed in RLT+ β-mercaptoethanol buffer and stored at -20ºC for subsequent RNA analysis.

The qPCR analysis was based on analyzing differences between the whole blood leukocyte fraction (white blood cell; WBC) and enriched CTC fractions (with and without *in vitro* incubation). The following TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, U.S.) were used for gene expression monitoring in all samples: TA genes, including ACTB (control), CD24, CD44, CD45, CD68, KRT19, EpCAM, MUC1, MGB, HER2, ESR, and PGR, as well as CA genes, including MRP1, MRP2, MRP4, MRP5, MRP7, MDR1, and ERCC1.

Based on gene expression analysis, CTC-enriched samples with elevated relative expression levels in two or more TA genes were considered CTC-positive when compared to their matched WBC samples.

Statistical analysis

The data were compared by standard tests using GraphPad Prism software vs. 9.1:0 (GraphPad Prism, U.S.). The evaluation of qPCR data was based on the standard ddCT method (55). qPCR results were analyzed using GenEx Professional software (MultiD, SE), which enabled multifactorial comparisons between the involved groups. Relative RNA levels are shown graphically in clusters (Supplementary files SF2 Methods). The differences between the samples were compared by the U Mann-Whitney test (significance level at $p < 0.05$ if not set automatically by GenEx).

Results

CTCs and tumor volume regression during NACT

The effects of NACT were clinically evaluated by bimanual breast palpation and ultrasound examination at the time of blood collection for CTC tests. Regression of the tumor mass was assessed as significant (response rate - 3, RR=3) if the tumor volume was reduced by more than 50%, as moderate (RR=2) if the degree of regression was 50%, and as minimal (RR=1) if the degree of regression was less than 50%. For non-significant tumor regression or even progression, a response rate equal to zero (RR=0) was applied. Patients with RR= 2 and 3 were assigned as responders in the further analyses.

The analysis of tumor volume reduction following the administration of AC vs. TAX during NACT showed that AC therapy was assessed as responsive in 60% (12/20) of tumors compared to TAX in 33.3% (6/20) (n.s.). The complex NACT effect was significant in 70% of patients in the tested cohort (14/20). Table 2 shows more details. The response rate was slightly higher in HER2-negative tumors when compared to HER2-positive tumors (64.2% vs. 50%; n.s.). 75% responsiveness was reported for TNBC (Table 3).
CTCs were detected in 85% of patients (17/20) prior to NACT, in 88% of patients after AC therapy (16/18), in 72% of subjects after TAX therapy (12/18). No significant difference was found between the groups in terms of the presence of CTCs when the group of non-responders was compared to the group of responders. However, 100% CTC positivity was found for the non-responder group after NACT as compared to 61.5% CTC positivity in the responder group (Table 2).

After completing NACT, reduction in CTCs was observed in more HER2-negative tumors compared to HER2-positive tumors (42.9% vs. 16.6 %) (n.s.) (Table 3).

During the follow-up period (185-2455 days, mean 1862 days), 8/20 patients progressed (40%). During NACT, all of these patients were positive for CTC in all tests. The CTCs obtained from these samplings expressed an excessive number of CA genes (≤ 4). **There was a significant difference in comparison of PFS rates between the group with the expression of more than four CA genes and the group with the expression of fewer than four CA genes during NACT (p=0.024).**

During NACT, the presence of CTCs (3 consecutive tests for each patient) identified patients with relapses and indicated significantly shorter PFS rates (p= 0.03). Tumor volume reduction was also significantly indicative for progressive disease (p = 0. 0224).
Overall, NACT did not significantly affect CTC positivity in the samples.

After NACT, CTCs were still present in 70.5% (12/17) of patients (responders vs. non-responders, 100% vs. 61.5%; n.s.).

CTCs released from the HER2-negative tumors expressed HER2 in 29% of all samples collected during the whole follow-up period (16/55) and in 32% of samples from NACT follow-up (10/31). The change accounted for 78.5% of HER2-negative patients (11/14) in total. 63.6% of the conversion cases occurred during NACT (7/11). For the remaining 4 patients (36.3%), conversion to HER2+ CTCs occurred later during adjuvant therapy.
During NACT, CTC reduction was observed in 42.9% of the HER2-negative patients, but only in 16.6% of the HER2-positive BC patients. Relapses were also more prevalent in the HER2-positive patient group (28.5 vs. 66.6% n.s.)
Complex information on NACT effects reflected in captured CTCs is shown in detail in Table 5 and Supplementary File ST1. Development of chemoresistance during NACT and adjuvant treatment is given in detail in Table 5 and Supplementary file SF Patient Follow-ups. The proportion of chemoresistant CTCs in the CTC fraction was related to worse therapy outcome. Resistant patients had elevated MRP1 gene during AC therapy and MRP1 and MRP7 genes during and after TAX therapy (Table 5).

Changes in primary tumor characteristics and CTCs during NACT

After completing NACT and surgery, definitive histology was compared with the pre-treatment biopsy (Supplementary file ST1). In most cases, the effect of NACT was linked to a significant decrease in Ki67. Only in 2 cases, the decrease in Ki67 was very small or there was no decrease in Ki67 (patients no 9 and 14). The BC phenotype changed significantly in one patient (no 9; pregnancy during NACT). We hypothesize that the loss of ER and PR expression could be caused by termination of pregnancy. Insufficient duration of NACT and AC monotherapy could be the cause of a small decrease in Ki67.

The loss of ER/PR+ expression under the influence of NACT is reported in the Supplementary file ST1. The distribution of primary tumors i.e., ER+, HER2+ and TNBC was as follows: 25%, 30% and 45%, respectively. The discordance in ER and HER2 expression in primary tumors and CTCs is given in Tables 4.1 and 4.2.

The distribution of CTCs during NACT was discordant in patients with ER-positive primary tumors (only 8 % CTCs were ER+). During NACT, 80% of samples from HER2+ patients showed increased HER2-negative expression. Interestingly, the phenotypic evaluation of CTCs during NACT showed HER2+ CTCs in TNBC in 32% and in 29% of samples during the follow-up. In HER2+ primary BC, discordance in HER2 status in CTCs was observed in nearly 50% cases. Therefore, in these patients, CTCs predominantly retained more aggressive properties. Tables 4.1 and 4.2 show more details on ER/HER 2.

Monitoring of BC treatment during NACT and after the surgical period

During the follow-up (1-5.5 years), we observed relapse in 8 patients, 6 of whom died. In our group of patients, the 1-year OS was 95 % (1/20 died), the 2-year OS was 78 % (3/20 died) and the 5-year OS was 70% (6/20 died).

The follow-up of all patients enrolled in the study is shown in Supplementary File SF1 Patient Follow-ups. The follow-up protocols include reports on the presence and characterization of CTCs during disease monitoring. Specific attention was paid to patients who achieved pCR after NACT.

Figure 1 shows the case of a young patient (no. 7) with LABC (medullary character). She underwent standard AC and TAX therapy with very good clinical effect (RR= 3), which was reported after TAX therapy. Although CTCs displayed no markers of chemoresistance before therapy, the expression was present during and after AC (the former with multi-resistant and the latter with AC-resistant CTC phenotype). Interestingly, HER2+ and ESR1 (ER) expressing CTCs were present in either both or one of the later blood draws, respectively.

The effect of TAX was confirmed both during the histological examination and MRD and the results showed pCR and CTCs negativity. As the primary tumor was ER-positive and PR-positive, the patient was still on adjuvant hormonal therapy. The first follow-up blood analysis conducted 5 months after surgery did not show CTCs, but the tests performed later (12 months and 15 months postoperatively) revealed MRD based on the presence of CTCs. Our data suggest that CTCs were not sensitive to hormonal therapy given by their increased expression of chemoresistance markers. Although the patient remained in clinical remission, the persistence of chemoresistance in CTCs warrants close monitoring.

Discussion

NACT is a standard approach in therapy of LABC and CTCs are believed to be associated with tumor aggressiveness. Studies have reported a lower prevalence of CTCs in PBC compared to MBC with positivity rates ranging from 22 to 23 % before NACT, from 10 to 17 % after NACT (56-58) and from 19 to 43 % in the adjuvant setting (59-62). Lavrov et al. detected CTCs in 38% of patients with early triple-negative disease and 42% of triple-negative LABC (63). By using multi-cytokeratin-specific antibodies to detect CTCs, Serrano et al. observed CTCs in 70 % of patients before NACT and in 54% of subjects after NACT (64). Camara et al. reported an even higher frequency of 83% in patients prior to NACT (65).

Pierga et al. detected CTCs in 39% of patients before the start of therapy and a rapid decrease to 9% after four cycles of chemotherapy (66). CTC analysis conducted by Sabatier et al. revealed 25 % and 8.9 % of CTCs-positive patients at inclusion and after one cycle of therapy, respectively (67).

In our study, CTCs were detected in 85 % of patients before NACT and 70.5 % of subjects after NACT. The relatively high detection rate of CTCs in our cohort can be explained by a high prevalence of clinical risk factors (95% of young premenopausal woman, 75% of HER2+ and 45% of TNBC, 100% of tumors with high Ki67 and 80% patients with G3, and 75% of patients with LABC with lymphatic node involvement).

Another explanation of high CTC detection rates may be the uniqueness of the CTC enrichment method used in the study protocol. The two-step detection protocol (MetaCell®) combining size-based filtration with cytomorphological and molecular characterization may identify more CTCs that go beyond the limited epithelial definition based only on EpCAM and cytokeratin expression (e.g. of 105 CTC samples analyzed by qPCR, increased expression of EpCAM was confirmed in only 16 cases (15.2%), whereas the expression of KRT 18/19 was documented in 90 (85.7%), HER2 in 34 (32.5%), MUC1 in 31 (29.5%) and MMG in 12 samples (11.4%).

The presence of CTCs before or after chemotherapy was associated with worse outcome compared to patients who were persistently CTC-negative in the SUCCESS trial (68).

Similarly, in the phase II AVASTEM trial, CTC detection at baseline was a prognostic marker for BC (NACT-bevacizumab combination independently of tumor response) (67). Many studies have clearly demonstrated a prognostic impact of CTCs not only in MBC but also in early BC (69-71).

In our study, patients remained CTC-positive during NACT (85 % of CTC+ before NACT, 88% of patients after AC, 70.5% of subjects after NACT). It is expected

that under the influence of NACT, tumor cell mobilization occurs and the number of released proliferating CTCs decreases. On the other hand, CTCs released from the primary tumor induced by NACT can lead to an increasing number of CTCs or their fragments detected in the circulation. Additionally, it was shown that systemic response to treatment was independent of local response (72).

Our analysis showed that TNBC tumors were more sensitive to NACT and the effect of AC was more significant in these tumors than the effect of TAX when tumor volume reduction was compared.

Unfortunately, we cannot confirm the correlation between tumor shrinkage and the decrease in the number of CTCs due to the methodological limitations of MetaCell®.

However, NACT responders were found mainly in the group of patients with CTCs expressing epithelial markers and CTCs with a minimal CA-gene expression. CTC negativity during NACT was found only in patients with the documented clinical effect (tumor volume reduction measured by ultrasound). In NACT non-responders, two or more CA-genes were usually frequently overexpressed in the CTC fraction.

To answer the question of how long NACT should be administered in the case of permanent presence of CTCs, it needs to be elucidated whether the presence of CTCs has a more prognostic or predictive value.

pCR is usually defined as the absence of invasive and non-invasive carcinoma in breast tissue. In the presented study, HER2+ tumors responded in 50% of cases, whereas TNBC responded in 75% (6/8) of cases. If we assume that aggressive cancer cells and CTCs may exist in these subtypes, we could hypothesize that pCR could be explained by the eradication of highly proliferating tumor cells (sometimes in significant numbers). In both aspects, the number and the characteristics of tumor cells could be equally important. Based on our observations, we conclude that CTC characterization could significantly supplement the information on the number of CTCs.

Several neoadjuvant clinical trials in BC assessed the benefit of combining additional chemotherapy (e.g. capecitabine) or targeted therapy (e.g. bevacizumab) with a standard AC or TAX-based chemotherapy regimen. Although the results of combination regimens in HER2-negative tumors are unconvincing (12-14), targeted anti-HER2 therapy added to standard NACT improved significantly the outcomes for HER2-positive patients (15-17). In these studies, a higher incidence of pCR in ER-/HER2+ (HER2-enriched) subtype was also shown compared to ER+/HER2+ BC. For patients with TNBC who participated in the CALGB and GeparSixto trials, significant improvement in pCR rates in the breast and axilla was demonstrated when carboplatin was added to more complex neoadjuvant AC- and TAX-based regimens (18,19). In the GeparTrio trial (20), the benefit of switching

NACT in non-responding patients was evaluated. Patients with no clinical response to neoadjuvant TAC (docetaxel, doxorubicin, cyclophosphamide) were randomized to four additional cycles of the same regimen or four cycles of vinorelbine and capecitabine. Although the benefit for ER+ patients was outlined (DFS, OS), future studies are warranted to specify treatment according to the molecular profile of the disease. Thus, the need for new biomarkers reflects the shortcomings of existing therapy options. Although AC and TAX regimens in NACT are considered standard clinical practice, recurrence due to clonal expansion and/or resistance of residual tumor cells due to treatment selection pressure occurs.

Clinical trials showed that NACT has the same benefit for patient prognosis as adjuvant therapy. It can be assumed that the higher volume of MRD in patients treated with NACT should have no effect on the risk of disease relapse. On the other hand, the prognostic value of CTCs is based on their rate in blood. However, detailed studies should be performed to find the connection between primary disease and the prevalence of CTCs.

In our study, pCR after NACT was achieved in 4/9 TNBC patients. Despite pCR in the case of TNBC patients, all of their blood samples were assessed as CTC-positive. The validation of prognostic significance of pCR is needed before we can say that the eradication of CTCs could be a new goal of treatment instead of pCR.

CTCs can survive as non-proliferating, dormant cells, and are associated with higher resistance to chemotherapy (73). After the completion of NACT, we found a few cases with persistent multi-resistant CTCs refractory to therapy (CTC – samples were positive for more than 1 chemoresistance gene in 7/20 cases, 35%). Considering the higher significance of CTC characteristics, the question of how to treat resistant disease arises. One option could be related to the use of CTC-targeted therapy, while another one could be connected with the removal of the primary tumor rather than random systemic treatment.

The significance of the status of CTCs after definitive treatment and their long-term persistence was assessed in many studies. The results obtained in the SUCCESS trial suggested CTC detection after chemotherapy was associated with shorter DFS and OS (74).

Van Dalum et al. reported a significantly shorter time to disease recurrence and death in women with detectable CTCs 1, 2 or 3 years after the end of adjuvant therapy or radiotherapy compared to CTCs-negative patients (75). Similar results were also obtained by Trapp et al. (76). Sparano et al. observed a 13-fold higher risk of recurrence in patients 5 years after diagnosis and without clinical evidence of disease when at least 1 CTC was detected at late time point (77).

Negative results of the SWOG 0500 study could be based on the lack of CTC characterization. We found a significant discordance in ER and HER2 status

compared to primary disease and MRD. HER2+ primary tumors retained HER2+ CTCs, mainly during NACT, after which they lost sensitivity to anti-HER2 therapy, very often during adjuvant trastuzumab therapy. HER2 status changed very dynamically and CTCs retained aggressive properties in HER2-negative tumors. Up to 23% of the CTCs samples in HER2-negative tumors were confirmed to be HER2-positive. This percentage was found in 11 of 14 HER2-negative patients. This number should be highly considered in the context of HER2 aggressiveness.

The comparison of responders and non-responders (patients developing disease relapse during adjuvant therapy) at the molecular level could complete our knowledge related to the significance of CTCs in the post-operative follow-up of patients as documented in the Supplementary file SF1 Patient Follow-ups. Prolongation of adjuvant hormonal therapy was shown to have clinical significance, confirming that the persistence of MRD exists long after surgery and requires longer treatment.

We hypothesize that simultaneous CTC monitoring could predict the risk of disease relapse earlier and more accurately compared to standard tumor markers and imaging studies. Therefore, both the number and CTC characteristics can play a critical role.

As CTC-guided therapy is not clinically indicated today, CTCs could be used only for treatment response monitoring. Promising designs of new studies focused on the predictive value of CTCs and studies of large numbers of patients are the main prerequisite for defining the predictive value of CTCs (78-83). CTCs could be used as a tissue source for testing sensitivity to different drugs in the future, which is highly attractive (84).

Conclusions

The relationship between CTCs and the response to tumor therapy was analyzed from the perspective of different tumor histopathology. We believe that monitoring of the presence of CTCs during NACT, including gene expression analysis of TA genes and CA genes, could identify patients at continuous risk and predict therapy outcomes. CTCs in patients with non-responding tumors expressed an excessive number of genes associated with chemoresistance.

Ultrasound monitoring alone is perhaps insufficient, whereas the combination of ultrasound and CTC monitoring could prove to be beneficial and enable earlier detection of disease relapse. Therefore, in cases of non-responding tumors based on the volume and the presence of chemoresistant CTCs, it may be more effective to reconsider the duration of NACT and possibly to switch from AC to TAX or to discontinue NACT and proceed to surgery. There are no data on the prolongation of NACT beyond the standard duration. The results from adjuvant trials with metronomic dosing of chemotherapy or adjuvant systemic treatment during NACT

may clarify the connection between persistent MRD and patient outcome. We believe that CTC-targeted therapy may improve patient outcome even if used only for monitoring therapy response. Until the end of prospective trials targeting the predictive value of CTCs, the utility of CTCs and CTC-related information are limited to palliative indications or support adjuvant therapy choice (i.e., indication for adjuvant chemotherapy in TNBC to treat residual disease after NACT).

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The Ethics Committee of the University Hospital Kralovske Vinohrady Prague approved the study protocol according to the Declaration of Helsinki (decision EK-VP/32/02014). All patients provided written consent.

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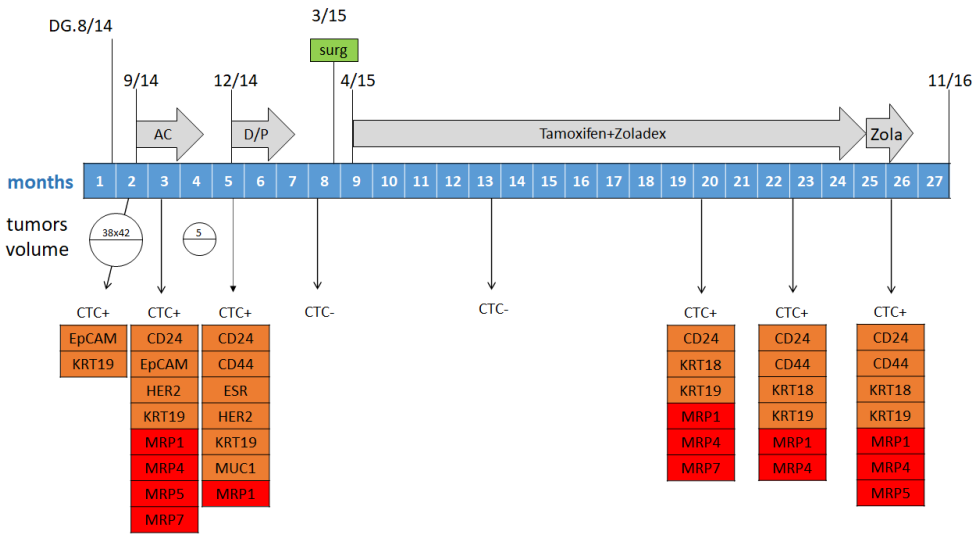
Figure 1. The schematic study protocol of patient no. 7

The disease course, therapy administration (neoadjuvant, adjuvant period) and circulating tumor cell (CTC) sampling are documented over the follow-up period. Gene expression testing was performed for positive CTC samples. Genes listed under the CTC+ had higher expression in enriched CTC fraction than in paired white blood cell fraction. The figures clearly show the evolution of resistant cancer cell clones later during adjuvant therapy (Tamoxifen with Zoladex). Personalized graphic protocols for each patient in the study are included in the Supplementary file SF 1 Patient Follow- up.

Patient no. 7	Clinicopathological features	
Age: 31	Pre-surgery	Post-surgery
TNM	T2N1M0	pCR
Receptors	ESR50, PGR10, HER2-	pCR



AC – doxorubicin+cyclophosphamid; D/P – docetaxel/paklitaxel; surg – surgery; Zola - Zoladex

13.2. Publication II

Breast Cancer Res Treat (2017) 166:695–700
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PRECLINICAL STUDY

Molecular characterization and heterogeneity of circulating tumor cells in breast cancer

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Abstract

Introduction This study analyzes peripheral blood samples from breast cancer (BC) patients. CTCs from peripheral blood were enriched by size-based separation and were then cultivated in vitro. The primary aim of this study was to demonstrate the antigen independent CTC separation method with high CTC recovery. Subsequently, CTCs enriched several times during the treatment were characterized molecularly.

Methods Patients with different stages of BC ($N = 167$) were included into the study. All patients were candidates for surgery, surgical diagnostics, or were undergoing chemotherapy. In parallel, 20 patients were monitored regularly and in addition to CTC presence, also CTC character was examined by qPCR, with special focus on HER2 and ESR status.

Results CTC positivity in the cohort was 76%. There was no significant difference between the tested groups, but the highest CTC occurrence was identified in the group undergoing surgery and similarly in the group before the start of neoadjuvant treatment. On the other hand, the lowest CTC frequencies were observed in the menopausal patient group (56%), ESR+ patient group (60%), and DCIS group (44.4%). It is worth noting that after completion of neoadjuvant therapy (NACT) CTCs were present in 77.7% of cases. On the other hand, patients under hormonal treatment were CTC positive only in 52% of cases.

Discussions Interestingly, HER2 and ESR status of CTCs differs from the status of primary tumor. In 50% of patients HER2 status on CTCs changed not only from HER2+ to HER2−, but also from HER2– to HER2+ (33%). ESR status in CTCs changed only in one direction from ESR+ to ESR−.

Conclusions Data obtained from the present study suggest that BC is a heterogeneous disease but CTCs may be detected independently of the disease characteristics in 76% of patients at any time point during the course of the disease. This relatively high CTC occurrence in BC should be considered when planning the long-term patient monitoring.

Keywords CTCs · Circulating tumor cells · Breast cancer · Cultivation · In vitro · MetaCell · Gene expression

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Introduction

Enumeration of circulating tumor cells (CTCs) has showed a prognostic role in various stages of the breast cancer (BC). Hormone receptors (estrogen and progesterone) and HER2 status of primary BC tumor have been established during standard clinical biopsies and are of crucial importance in the choice of treatment. Real-time tumor monitoring through CTC enumeration could be an important indicator of individual cancer development [1].

CTCs as biomarkers can offer some valuable information about a patient's tumor, if detection, separation, and characterization are performed in a reliable manner. Although occurrence of CTCs in patients' peripheral blood is often very low, enrichment methods can be introduced for CTC separation before their characterization. They are usually based on surface protein expression, size, density, electric charges, or deformability of CTCs.

This study analyzes peripheral blood samples from patients with BC. CTCs from peripheral blood were enriched by size-based separation and then cultivated in vitro. The primary aim of this study was to demonstrate the antigen independent high sensitive separation method and a possibility of molecular characterization of CTCs enriched several times during the treatment.

Materials and methods

Patients

To date 167 patients with diagnosed BC have been enrolled in the study in accordance with the Declaration of Helsinki. All patients were candidates for surgery, surgical diagnostics, or with planned or applied chemotherapy. Based on their informed consent, clinical data were collected from all participating patients. Basic cytopathological data are reported in Table 1. For each patient, approximately 2 × 8 mL of venous blood was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 h after the blood draw.

Table 1 Basic cytopathological characteristics of patients

	N	(%)
Stage		
0	3	2
IA	45	30
IIA	64	42.7
IIB	20	13.3
IIIA	13	8.7
IIIB	1	0.67
IIIC	4	2.67
Histopathological features		
Benign	2	1.7
DCIS	9	7.6
LCIS	1	0.85
IDC (NST)	76	65.6
ILC	14	11.86
Mixed	16	13.6
Menopausal status		
Premenopausal	65	39.39
Menopausal	18	10.9
Postmenopausal	82	49.7
Tumor size		
T1	63	61.1
T2	36	34.9
T3	4	3.8
Nodal involvement		
N0	56	56.5
N1	37	37.3
N2	6	6
Grading		
G1	7	11.8
G2	24	40.6
G3	28	47.4
HR and HER2 status		
HR+ HER2+	16	11.7
HR− HER2+	7	5.1
HR+ HER2−	91	66.4
HR− HER2−	23	16.8

CTCs enrichment and culture

The recently introduced size-based separation method for viable CTC enrichment process (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) [2–6] is based on the filtration of peripheral blood through a porous polycarbonate membrane (with pores of 8 μm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL of fluid. The standard 8 mL peripheral blood sample from patients suffering from BC was transferred into the filtration tube. Gradual transfer of the blood in several steps is preferred to prevent blood clotting on the membrane filter. The peripheral blood flow is supported by capillary action of the absorbent touching the membrane filter. The filtered CTCs were observed immediately after filtration on the membrane. The control and presence of filtered CTCs immediately after isolation eliminates false negative results. The membrane filter is

kept in a plastic ring that is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and CTCs are cultured on the membrane in vitro under standard cell-culture conditions (37 °C, 5% atmospheric CO_2) and observed by inverted microscope. The CTCs were grown in FBS-enriched RPMI medium (10%) for a minimum of 14 days on the membrane. Alternatively, the enriched CTC fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be translocated on a microscopic slide. Microscopic slide is preferred if immunohistochemistry/immunofluorescence analysis is planned. If an immediate CTC analysis is awaited, the CTC fraction is transferred in PBS (1.5 mL) to a cytospin slide. The slide is then dried for 24 h and analyzed by histochemistry (May-Grünwald staining) and/or by automated immunohistochemistry protocols (Ventana, Benchmark Ultra, Roche) using standard differential diagnostic antibodies in the pathological evaluation process.

Cytomorphological analysis

The stained fixed cells captured on the membrane were examined using light microscopy in two steps: (i) screening at ×20 magnification to locate the cells; (ii) observation at ×40/×60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized, and the images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size ≥10 µm); (ii) irregular nuclear contour; (iii) visible cytoplasm, cells size over 15 µm; (iv) prominent nucleoli; (v) high nuclear-cytoplasmic ratio; (vi) proliferation; (vii) actively invading cells creating 2D or 3D cell groups.

Gene expression analysis (GEA)

The key purpose of GEA was to compare gene expression of tumor-associated markers in the CTC-enriched fractions to that in the whole blood (white blood cells). Gene expression analysis can be performed to confirm the origin of the captured cells on the separation membrane. Gene expression analysis (GEA) allows up to 20 tumor-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostics markers for qPCR test are chosen in accordance with the expected diagnosis.

RNA is isolated from the whole blood and CTC-enriched fraction on the membrane. The CTC-enriched fraction of cells grown on the separation membrane in vitro (the so-called "membrane fraction") was used for RNA isolation.

Finally, CTC-gene expression analysis allows identification of the relative amount of tumor-associated (TA) markers in the whole blood and in CTC-enriched fractions. If the tumor-associated genes are highly expressed in the CTC fraction, a subsequent analysis of chemoresistance-associated (CA) genes is performed. Molecular analysis helps to identify which type of chemotherapeutic agents may be of use in tumor therapy and assigned as personalized cancer therapy based on CTC.

The cells captured on the membrane are lysed by RLT-buffer with beta-mercapto-ethanol (Qiagen). RNA is then isolated using the RNeasy Mini Kit (Qiagen). RNA from the whole blood is isolated with a modified procedure and the quality/concentration of RNA is measured by NanoDrop (ThermoScientific). As there are only up to a few hundred cells on the membrane, the median concentration of RNA is quite low (5–10 ng/µl). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA production. Gene expression analysis was performed using Taqman chemistry with Taqman MGB-probes for all the tested genes (Life Technologies).

The following genes associated with tumorigenic character and therapeutic potential in breast cancer were chosen for the multimarker GEA panel: ACTIN, CD45, CD68, EPCAM, MUC1, KRT18, KRT19, ESR, PGR, MAMMAGLOBIN, HER2, CD24, CD44. Additionally, genes associated with chemoresistance were tested (MRP1-10, MDR1, ERCC1).

Statistical analysis

All analyses were performed using clinicopathological information transformed into variables 0 and 1 if applicable for tested characteristics. Chi squared test, *t* tests, cluster analysis, and correlation analysis of qPCR data were outperformed using GeneX (MultiD, SE) and GraphPadPrism versus 5 (Graphpad, US). *P* value of less than 0.05 was considered statistically significant.

Results

The main focus of the study was to detect CTCs shortage in BC patients by a new methodological approach which is based on size-dependent separation of CTCs and subsequent cytomorphological evaluation. Cytomorphological evaluation using vital fluorescence microscopy approach (Fig. 1) enables further use of the viable captured cells for RNA/DNA analysis.

Patients diagnosed with different stages of breast cancer (BC) ($N = 167$) were included into the study. The patients were divided based on clinicopathological criteria and CTC presence was tested. Summary of the collected CTC positivity data is presented in Table 2.

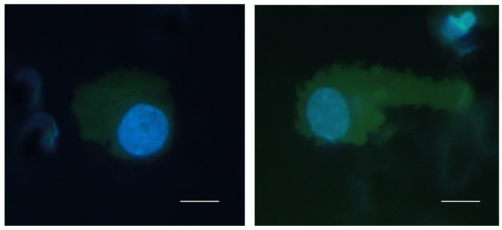

Fig. 1 CTCs isolated from a patient with breast cancer, captured on the separation membrane (vital fluorescent staining— NucBlue® and Celltracker®). *Bar* represents 10 μm

CTC positivity in tested cohort was 76%. There was no significant difference between tested subgroups, identifying a possible CTC presence, but the highest CTC occurrence was observed in the group undergoing surgery (86.6%) and similarly in the group before the start of neoadjuvant and adjuvant treatment (82.3%).

It is important to comment on relatively high CTCs presence even after neoadjuvant therapy has been completed (77.7%). It can be assumed that in these patients therapy did not diminished all the cancer cell types.

There were no significant differences in CTC frequencies observed based on stage definitions. Considering the histopathological character of the primary tumor, the lowest CTC positivity was observed in DCIS (44.4%). Relatively low CTC frequency was observed in the menopausal patient group (55.5%).

Furthermore, it can be concluded that in tumors with ESR expression (ESR+) and without PGR expression (PGR-) CTCs were detected only in 60% (9/15) of tested cases, whereas in ESR+/PGR+ tumors CTC positivity was 73% (68/93). On the other hand, in patients with ESR-negative tumors CTCs were detected in 96.7% which is almost all of the patients under study (30/31). Therefore, it must be mentioned that during the therapy only 52.9% (9/17) of patients exhibited CTCs. Nevertheless, menopausal stage has to be considered if ESR/PGR expression is evaluated. The correlation of the menopausal status and ESR/PGR expression is illustrated in Fig. 2 which shows that hormonal receptor-positive tumors exhibit the lowest CTC detection frequencies in comparison to the HR-groups.

Similarly, even if not statistically significant, it can be seen that HR +/HER2− tumors, irrespective of the menopausal stage show the lowest CTC frequency rates (see Fig. 3).

In parallel, 20 patients were monitored regularly during the course of the disease and in addition to CTC presence, CTCs character was also examined by qPCR with special focus on HER2 and ESR status. In total 43 qPCR analysis were evaluated. Therapeutically, the most relevant findings are as follows: HER2 and ESR status of CTCs may differ from the status of primary tumor.

Table 2 CTC positivity identified in BC—patient subgroups

	N	(%)
CTC Positivity	CTC+	
CTC+	**119**	**72.1**
CTC−	**46**	**27.9**
Stage		
0	3	100
IA	31	68.9
IIA	47	73.4
IIB	16	80
IIIA	10	76.9
IIIB	0	0
IIIC	4	100
Histopathological features		
DCIS	4	**44.4**
LCIS	1	100
IDC (NST)	55	72.4
ILC	13	92.9
Mixed	9	56.3
Menopausal status		
Premenopausal	51	78.4
Menopausal	10	**55.5**
Postmenopausal	58	70.7
Tumor size		
T1	63	74.6
T2	36	88.8
T3	4	75
Nodal involvement		
N0	23	82
N1	17	78
N2	2	66
Grading		
G1	**5**	70
G2	14	58
G3	23	82
HR and HER2 status		
HR+ HER2+	13	81.3
HR− HER2+	7	100
HR+ HER2−	64	**70.3**
HR− HER2−	22	95.7
ESR+ PGR+ vs. ESR+ PGR−		
Therapy		
Before therapy	28	82.3
During HT	9	**52.9**
After NACT	7	77.7
Before surgery (after biopsy)	39	86.6

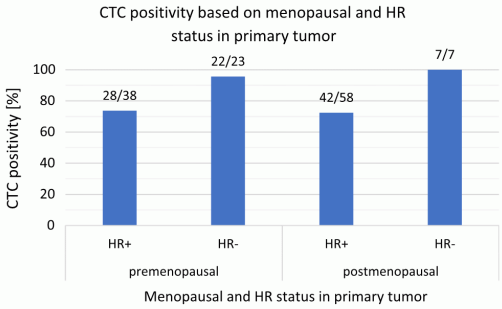

Fig. 2 CTC positivity in relation to menopausal stage and primary tumor HR− expression

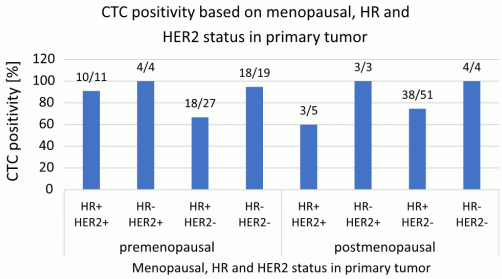

Fig. 3 CTC positivity in relation to menopausal stage and primary tumor HR and HER2− expression

The most frequent changes were seen in the triple negative BC (TNBC) group ($N = 12$) where 27 samples were evaluated. HER2 presence was confirmed in CTCs in four cases, which means that the change from HER2– to HER2+ occurred in 15% of tested samples, but that these four changes can be ascribed to four different patients. The change was relevant for four out of 12 patients (33.3%) which is already a significant number. Similarly, in 50% of patients, HER2 status changed from HER2+ to HER2− (3/6).

ESR status in CTCs changed only in one direction from ESR+ to ESR− (3/3). These patients' primary tumors were diagnosed as ESR+/PGR+/HER2−. This group of patients will most probably exhibit very frequent changes.

Taken together, due to relatively high numbers of CTC positivity in different patient groups, we may conclude that a certain number of CTCs are always present in the blood of the patients. The cells have to be under selection pressure of treatment uninterruptedly. As soon as the selection pressure is stopped, new gene expression profile is displayed by CTCs.

The data obtained in the present study suggest that BC is a heterogeneous disease, but CTCs may be detected independently of the disease characteristics in 76% (119/165) of patients at any time point of the course of the disease. This relatively high CTC occurrence in BC should be considered in planning the long-term patient monitoring.

Discussion

Treatment decisions in BC are based on the characteristics of the primary tumor without considering the character of minimal residual disease or metastasis. However, tumors are evolving entities and genetic heterogeneity has been detected comparing the primary tumor with subsequent recurrences and metastases and analyzing different regions of the same tumor [7]. It has been hypothesized that the success of personalized treatments greatly depends on the capability to capture and monitor tumor heterogeneity over time and to consequently modulate therapies [8].

Detection and characterization of CTCs can contribute to the understanding of the disease and improved therapy monitoring as well as personalized treatment options. The key step is sensitive isolation and detection of CTCs. To date, various approaches have been also used to visually identify CTCs; however, the techniques employed to perform cell enrichment, immunohistochemical detection, and image analysis are complicated [9, 10]. Moreover, epithelial markers are currently used to detect CTCs; tumor cells, however, may lose their epithelial features during metastasis/dissemination or may not express these markers because of their heterogeneity [11]. Therefore, some CTCs could be unidentified during epithelial-mesenchymal transition (EMT) by the common CTC-enrichment strategies relying on epithelial markers [12]. According to recent findings, more invasive CTCs may lose their epithelial antigens as a result of the EMT process [13] and EMT has been increasingly recognized as the key mechanism of cancer drug resistance [14].

We have used a simple method, without any complicated processing steps, for detecting viable human CTCs in the peripheral blood by using physical features of CTCs. We believe that viable CTCs may be a less invasive, repeatable biomarker for monitoring tumor responses.

In our study more than 76% patients were CTC positive. This result provides evidence that BC cells migrate and disseminate from morphologically very early lesions. Hosseini et al. demonstrated that metastatic dissemination often occurs early during tumor formation [15]. Disseminated cancer cells detected in patients before the manifestation of breast-cancer metastasis contain fewer genetic abnormalities than primary tumors and indicate that dissemination occurs during early stages of tumor growth [16–19].

As demonstrated by the SWOG S0500 trial, the simple enumeration of CTCs is not sufficient to guide therapy [20]. There is increasing evidence that cancer evolves over

time because of its genomic instability and under the selection pressure of systemic treatments. These changes can be responsible for the appearance of drug-resistant clones. In studies of metastatic breast cancer (MBC), a discrepancy was observed between metastases or CTCs and the primary tumors in terms of HER2, estrogen and progesterone receptor expression [21, 22]. The loss of progesterone or estrogen hormone receptor expression in CTCs was described in 40% of receptor-positive MBC, while increased hormone receptor expression was detected in only 8% of triple negative MBC [21].

The clinical use of new CTC detection technique and the molecular characterization of isolated CTCs may lead to the development of personalized anticancer strategy in near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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13.3. Publication III

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Original Article

Circulating tumor cells: what we know, what do we want to know about them and are they ready to be used in clinics?

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Abstract: Circulating tumor cells (CTC) present in peripheral blood are assigned precursors of advanced tumor disease. Simplicity of blood withdrawal procedure adds practically an unlimited possibility of the CTC-monitoring and the advantages of the repeated biopsies over time. CTC got prognostic, predictive and diagnostic status with the technologic advance. Although the clinical utility of CTC has reached the high evidence, the significance of CTC testing was presented in the treatment strategy mostly with palliative intention. We report on the experiences with the CTC-testing in the CLIA-like laboratory working with the size-based CTC separation and *in vitro* culture. The data is presented in the form of case reports in patients with breast (BC), colorectal (CRC), prostate (PC) and lung cancer (NSCLC) to support the clinical utility of CTC during the neoadjuvant, adjuvant and palliative treatment. The presented findings support the evidence for liquid biopsy clinical implementation and enhance the ability of malignant disease monitoring and the treatment efficacy prediction.

Keywords: Circulating tumor cells, breast cancer, colorectal cancer, non-small-cell lung cancer, prostate cancer, chemoresistance

Introduction

The count of CTC in blood of oncological patients is very low [1]. Detection of CTC is also limited by their heterogeneity. Finally, the capacity of various malignancies to release CTC into the peripheral circulation is different depending on the stage of the disease and also on the type of malignancy [2-12]. The chance of CTC positivity is in general notably higher in metastatic than in the primary disease, e.g. 2-55% in primary BC vs. 40-80% in metastatic BC [13].

Technologies try to overcome the rare occurrence of CTC by using the enrichment step to separate CTC from blood cells. The negative selection is based on the elimination of leukocyte fraction (e.g. by using anti-CD45 antibodies) from blood. The positive selection utilizes surface features of CTC or their physical properties such as cells size or density. The most commonly used methods are based on the immunomagnetic selection of CTC with epithelial features. But these methods are impoverished to detect CTC with the lack of epithelial characteristics, e.g. cancer cells whose phenotype has been altered by the process of epithelial-mesenchymal transition (EMT) [14] or which have the character of stem cells [15]. Combinations of different methods and new approaches [16], in particular microfluidic systems, focus on increasing the sensitivity and specificity of CTC selection and detection.

The standardization of technologies at all levels of CTC identification and results interpretation based on different approaches is still a prob-

Table 1. Clinical indications to CTC examination

Prediction of disease response to neoadjuvant chemotherapy
Indication of "additional" adjuvant therapy in residual disease
Observation after adjuvant therapy
CTC monitoring after adjuvant therapy and during metastatic disease
CTC-testing after resection of metastases and early prediction of disease relapse
Assessment of KRAS mutation status from CTC
Strategy of using CTC for the palliative treatment guidance
Typing of tumors with unknown primary site or duplicate tumors

lem. The rare occurrence of CTC in non-metastatic disease is the reason why the threshold for the CTC positivity is different in the primary disease in comparison to the metastatic malignancy. Therefore the prognostic significance of CTC demonstrated by the use of various approaches has not the same weight.

The only FDA (U.S. Food and Drug Administration) approved method for the detection of CTC (Cellsearch®) is based on the separation of EpCAM (epithelial cell adhesion molecule) positive cells. Published data declare the prognostic significance of CTC detected by Cell search in metastatic BC [17], metastatic CRC and PC [2, 3].

The size-based enrichment protocol of CTC reported in our study enables capturing and *in vitro* cultivation of viable CTC (MetaCell®). CTC can be further analysed by the downstream molecular analysis (e.g. gene expression testing by qPCR). Previous studies indicated a fast and simple enrichment in various cancer types [4, 18-20].

In general, the prognostic significance of CTC is supported by several studies on the level of primary disease: in primary BC pre- and postoperatively [21, 22], in early CRC preoperatively [23], from the postoperative lavage of the peritoneal cavity [24] and after the adjuvant chemotherapy [25], or in early NSCLC [26].

The clinical utility of CTC is still the subject of clinical studies [27-30]. Well known SWOG s0500 trial did not support the assumption of the clinical benefit of early chemotherapy change in patients with the metastatic BC and persistent CTC after the first cycle of therapy [31]. The characterisation of CTC was not performed in this study so the "real" predictive significance of CTC was not considered. Conversely, the use of anti-HER2 antibody therapy in patients with metastatic BC and HER2-positive (HER2+) CTC resulted in the prolongation of the time to progression compared to the patients who were not treated with the targeted therapy [32]. CTC detection and characterisation in patients with metastatic castrate-resistant PC (CRPC) can select a group of patients who will most probably not benefit from the hormonal therapy [33]. Splice variant 7 of the androgen receptor (AR-V7) detected in CTC predicts tumor response to the hormonal therapy and taxanes. The patients with AR-V7 positive CTC treated with taxanes survived longer than those treated with the hormonal therapy [34].

The assumption is that the characteristics of CTC are more important than the total CTC number. Below presented individual case reports describe our experience with CTC examination in patients with BC, CRC, PC and NSCLC and the potential for their use in the clinical practice. Every case report is documented in the appropriate figure. The scheme of the disease course, patients' characteristics and results of CTC-testing are shown in pictures schematically. Questions asked by clinicians that lead to the indication of CTC-testing, are in relation to several therapy relevant points (**Table 1**). CTC examination was indicated only as an additional test to a standard diagnostics.

Material and methods

Before the blood collection, patients were informed about the purpose and nature of the examination and their agreement with testing was reaffirmed by the signing of a consent. Minimum of 7.5 ml peripheral blood was obtained (1.6 mg EDTA/1 ml of blood as anticoagulant) for the CTC examination once or repeatedly during 6-24 months.

Enrichment, cultivation and detection of CTC

Blood was subjected to the two-step analysis, consisting of the size- based capturing of cells (MetaCell®), the evaluation of cytomorphological parameters of captured and cultured viable cells by the fluorescent microscopy

Table 2. Genes associated with chemoresistance

Resistance to:	Genes associated with chemoresistance:						
Anthracyclines	MRP1	MRP2					
Taxanes		MRP2			MRP7		
Irinotekan/topotekan	MRP1	MRP2	MRP4				
Alkylating agents	MRP1	MRP2					
5-fluorouracil				MRP5			
Platinum derivates		MRP2		MRP5		ERRC1	
Metothrexat		MRP2	MRP4	MRP5			
Vinca-alkaloids	MRP1				MRP7		
Multi-drug resistance as defined by MDR1 (P-glykoprotein)							MDR1
Gemcitabine							RRM1/RRMM2

and by the gene-expression analysis (molecular detection).

CTC were enriched by the filtration of the peripheral blood using Metacell® separation tool (MetaCell s.r.o., Czech Republic). Captured cells were cultured *in vitro* under standard conditions (37°C, 5% CO_2) for 3-5 days. Subsequent cytomorphological analysis is based on the characteristics of captured and cultured cells stained by vital fluorescent dyes (Nucblue® Live Ready Probes® Reagent and CellTracker™ Green CMFDA, Thermo Fisher Scientific, USA). The cells were evaluated by standard cytopathologic criteria under the fluorescent microscope (e.g. cells size >15 μm, nucleus size >10 μm, irregular nuclear contour, high nuclear/cytoplasmic ratio, prominent and/or irregular nucleoli, cell proliferation presence, tri-dimensional cell sheets growth). The digital documentation of the captured cells is available for each patient. If the cells with CTC-character were detected, further molecular analysis was provided. CTC were lysed and stored in RLT-buffer with β-mercaptoethanol solution (-20°C).

RNA isolation and cDNA preparation

RNeasy Mini Kit (Qiagen, Germany) was used for RNA isolation from the frozen cell lysates (white blood cells (WBC) and CTC) stored in RLT-buffer. RNA quantity and quality has been checked by NanoDrop (Thermo Fisher, U.S.). High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific, USA) for cDNA synthesis using minimum of 100 ng RNA load for single cDNA reaction.

qPCR analysis

The gene expression of tumor-associated (TA) genes (disease specific), stem cell markers and control genes (ACTB) was evaluated. Additionally, the markers of white blood cells (CD45, CD68) were included. Subsequently, expression of genes associated with chemoresistance (CA-genes) was tested. The predictive associations of tested CA-genes MRP1, MRP2, MRP4, MRP5, MRP7, MDR1, ERCC1, RRM1, and RRM2 with the tumor chemoresistance are listed in **Table 2**. TaqMan™ Gene Expression Assays (Thermo Fisher Scientific, USA) were used for qPCR analysis in the samples analysed by PCR technology system Cobas® 480 (Roche s.r.o., Czech Republic).

The gene expression in particular cell-fractions (WBC, CTC) was evaluated for every patient individually. The gene expression in WBC fraction, CTC-enriched fraction and CTC-enriched and cultured fraction were compared to confirm the cancer cell presence. The qPCR data evaluation was based on $2^{-\Delta\Delta CT}$ methods used to calculate relative changes in the gene expression analysis [35]. Samples with relatively elevated expression of TA-markers (2 and more) in cultured CTC-fraction compared to WBC fraction were evaluated as CTC positive based on gene expression analysis. qPCR results were analysed by the means of GenEx Professional software (MultiD, Sweden) enabling multifactorial comparison (e.g WBC vs. CTC) applying Mann- Whitney test ($P<0.002$) in particular patients.

Clinical interpretation

The CTC-test result forms report on information about the presence/absence of CTC including the following statements: CTC presence was confirmed by the cytomorphological evaluation (YES/NO), CTC presence was confirmed by the elevated gene expression of the following

Age in time of dg	Staging before operation	Histology from biopsy	Staging after operation	Postoperative histology
34	T2N1M0	IDC, G3, TNBC, Ki67: 70%	T2N1aM0	Metaplastic carcinoma, G3, TNBC, Ki67: 70%

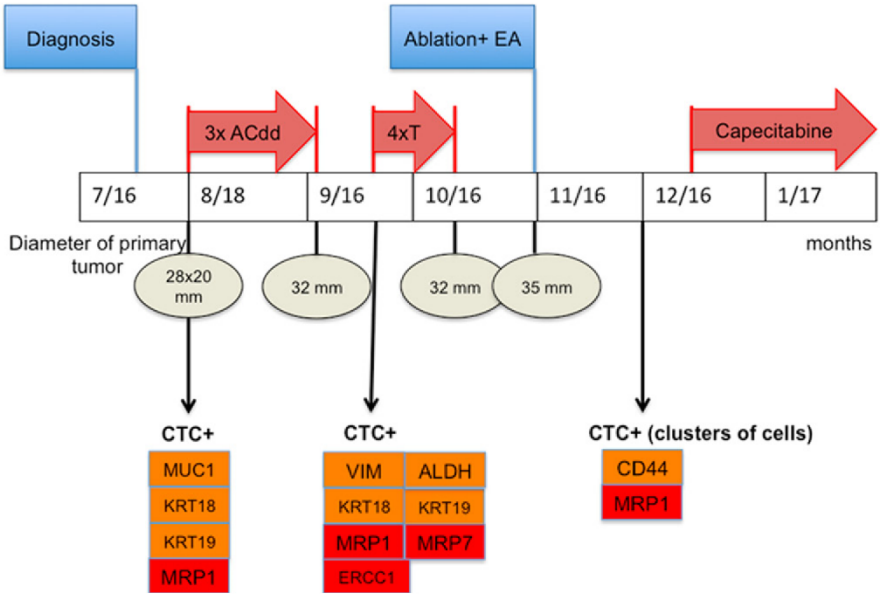


Figure 1. CTC monitoring during neoadjuvant chemotherapy in a patient with breast cancer. AC: doxorubicin + cyclophosphamide, dd: dose dense, T: paclitaxel, CTC+: CTC positivity, EA: axilla exenteration, dg: diagnosis, G: grade, TNBC: triple negative breast cancer, IDC: invasive ductal carcinoma, markers of stem cells: CD44/CD24, VIM (vimentin), ALDH (aldehyddehydrogenase), markers of epithelial cells: KRT18/19 (keratins), MUC1 (mucin), markers of chemoresistance: see **Table 2**.

genes: e.g. KRT18, KRT19, MUC1, CD24, HER2, ESR. The chemoresistance of CTC may be predicted by the elevated gene expression of the following genes: e.g. MRP2, MRP7. The combination of MRP2 and MRP7 may indicate a resistance to taxanes. MRP2 itself indicates a resistance to platinum-derivatives. MRP7 could be involved into a resistance against vinca-alkaloids too. Repeated measurements enable monitoring of dynamic changes on CTC in time.

Results

Clinical implementation of CTC-examination: Prediction of disease response to neoadjuvant chemotherapy (NACT) in a patient with BC (Case report 1)

Hypothesis: CTC monitoring during NACT may help to predict the early failure of the cancer therapy.

State of the art: To reveal non-responders based on clinicopathological parameters are not entirely possible. Conversely, CTC properties and, in particular the sensitivity to various cytostatics could be a predictor of the treatment response. The early shift of the chemotherapy regimen based on the evolving chemoresistance could boost the treatment efficacy. The characteristics of the primary disease usually do not correlate with the presence of CTC [36]. The detection of CTC provides additive prognostic and predictive information. The disease progression and the presence of CTC with the mesenchymal characteristics [37] could be the reason for the premature termination of NACT and the indication of a surgery. Whether the presence of CTC with mesenchymal features (stem cells like) can negatively influence the prognosis of the patients is not clear.

Predictive effect of pathologic complete remission is not exclusive, too [38].

Patient report (1): the response to NACT in triple negative BC (TNBC) patient (34 years old,

Age in time of dg	Staging before operation	Histology from biopsy	Staging after operation	Postoperative histology
53	T1cN1M0	IDC, G3, ER0, PR0,HER2 neg., Ki67: 40%	T1bN0(0/5)M0	NST, G1, ER10, PR0, HER2 neg., Ki67: 5%

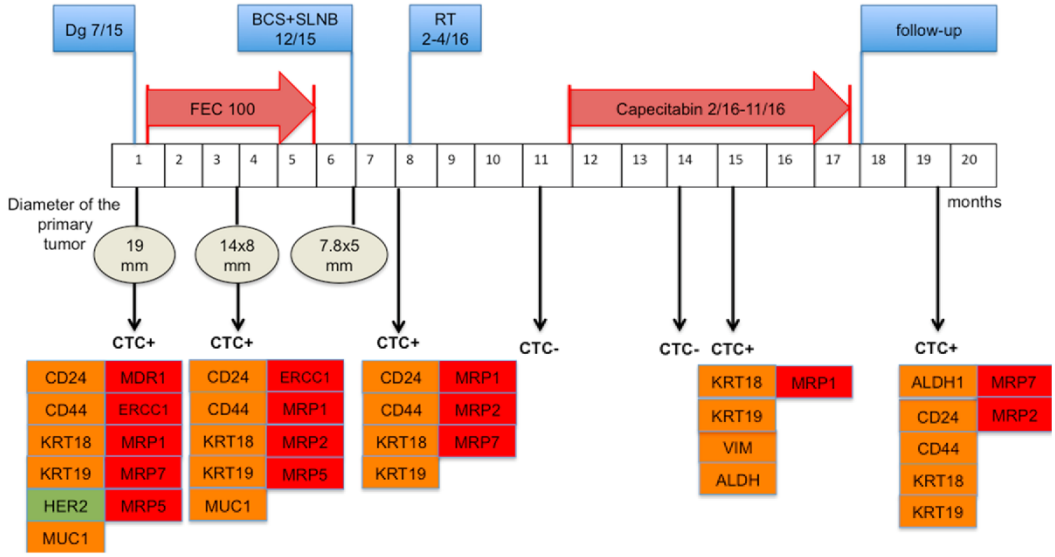


Figure 2. Indication of "additional" adjuvant therapy in a breast cancer patient with persisting CTC. CTC positivity: CTC+, CTC negativity: CTC-, FEC: fluorouracil, epirubicin, cyclophosphamid, RT: radiotherapy, BCS: breast conserving surgery, SLNB: sentinel lymphatic node biopsy, dg: diagnosis, IDC: invasive ductal carcinoma, G: grade, markers of stem cells: CD44/CD24, ALDH (aldehyddehydrogenase), VIM (vimentin), markers of epithelial cells: KRT18/19 (keratins), HER2: human epidermal growth factor receptor, MUC1 (mucin), markers of chemoresistance: see **Table 2**.

stage II) has been monitored. Tumor size was 28 mm at the beginning of the NACT; ultrasound examination described several pathological lymph nodes. CTC were present before NACT had started (**Figure 1**).

CTC displayed the expression of these TA-genes: MUC1, KRT18, KRT19 and CA-gene MRP1. After the 3rd therapy cycle with anthracycline (AC regimen), no therapeutic effect was observed by the ultrasound examination. CTC test was positive again and the level of tumor cells resistance spread (expression of MRP1, MRP7 and ERCC1 was elevated). Expression of MRP7 is associated with the prediction of taxane chemoresistance. Nevertheless, the patient received 4 cycles of paclitaxel in a weekly mode. According to the ultrasound imaging, the tumor size remained at 32 mm.

Subsequently, NACT was terminated and the patient was indicated for a surgery. The final histology described a metaplastic carcinoma (35 mm in diameter). The postoperative blood test detected clusters of CTC. The elevated expressions of keratins were no longer demonstrated but CD44 positive cells were present.

To be discussed: Due to the existing anthracyclines resistance (MRP1), the age of patient and the adverse outcome of NACT, the patient continues with the adjuvant capecitabine therapy (therapy choice is discussed below in the next case report).

Clinical implementation of CTC-examination: indication of "additional" adjuvant therapy (AT) in residual disease in a patient with BC (Case report 2)

Hypothesis: CTC molecular analysis during AT may help to predict the therapy efficiency and failure.

State of the art: AT administered after the removal of the primary tumor is one of the most difficult treatment strategies. The indication for the adjuvant chemotherapy (ACT) lacks person-

Age in time of dg	Staging of the disease	Histology
29	pT1c multi(3)pNO(0/2) M0	TNBC, G3, Ki67: 60%

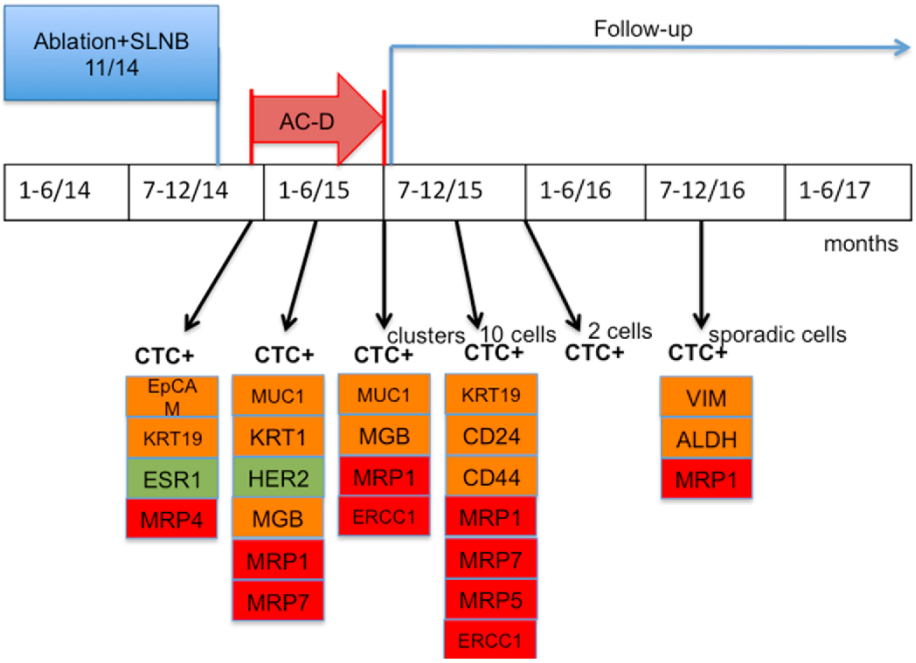


Figure 3. CTC monitoring during adjuvant therapy and in follow up period in a breast cancer patient. CTC positivity: CTC+, AC: doxorubicin + cyclophosphamide, D: docetaxel, SLNB: sentinel lymphatic node biopsy, dg: diagnosis, TNBC: triple negative breast cancer, G: grade, markers of stem cells: CD44/CD24, ALDH (aldehyddehydrogenase), VIM (vimentin), markers of epithelial cells: KRT18/19 (keratins), HER2: human epidermal growth factor receptor, ESR1: oestrogen receptor gene, MUC1 (mucin), MGB: mammaglobin B, markers of chemoresistance: see **Table 2**.

alization and endangers the patient with an unnecessary treatment and a possible ineffective therapy. The correct indication of the correct therapy requires further identification of the residual disease.

There is a lack of data for ACT indication beyond the standard length of the therapy. Create-X study [39] is the only study addressing this question, however, with many questions regarding not only the primacy of data [40, 41]. Nevertheless, an "additional" treatment strategy is requested in the clinical practice.

Molecular typing of TNBC divided this diagnosis into several subtypes with a different prognosis [42]. Treatment guidelines of TNBC have not accepted this fact yet, although the chemotherapy response differs in individual subtypes of TNBC [43]. The need for predictive markers in TNBC is therefore more than obvious and possible use of CTC is definite.

Patient's report (2): A case report of TNBC patient (44 years old, stage I) undergoing the additional ACT after the completion of NACT is reported (**Figure 2**). The ACT indication was based on the CTC persistence and primary disease residuum.

The first blood sample was tested before the start of NACT. Keratins (KRT18, KRT19), mucin (MUC1), human epidermal growth factor receptor (HER2) and MRP1 genes were overexpressed in the CTC-enriched fraction (for more details about CTC during NACT see **Figure 2**).

CTC persisted postoperatively, as well as their chemoresistant character. Although no CTC were present after RT, additional ACT with capecitabine was started. The presence of CTC

after the 4th capecitabine cycle was not confirmed. After the 6th capecitabine cycle CTC were detected again, furthermore the cells exhibited clustering and overexpressed markers associated with the mesenchymal character: vimentin (VIM) and aldehyddehydrogenase (ALDH1). We assume that the super-selection of the aggressive clone arose during the course of the capecitabine therapy. The expression of HER2 was seen only at the beginning of NACT. Because of the persistent sensitivity to the current treatment we continued up to 8 cycles of the capecitabine therapy. The patient is currently being monitored without any therapy and without any disease relapse.

To be discussed: In the presented case report we can demonstrate the aggressiveness of the tumor defined by persistent CTC long after the completion of the primary therapy and the possible therapeutic strategy of "watchful waiting" with the administration of systemic therapy apart from the completion of primary treatment. The indication of capecitabine according to the Create-X study is not entirely definite, as well as its inclusion into the AT treatment scheme in the period after the RT and in small T1b tumors. On the other hand, we know that the release of substantial quantities of CTC occurs early in tumors under 3 mm in the diameter [44]. Based on the observations in mice, the clusters of CTC have under observations in mice, 23-50 × higher metastatic potential, their presence thus predicts the ability of cells to establish secondary lesions [45].

Clinical implementation of CTC-examination: observation after AT in a patient with TNBC (Case report 3)

Hypothesis: CTC molecular analysis after AT may help to predict disease relapse.

CTC presence after the tumor resection and/or after the completion of AT predicts higher risk of the disease relapse. Since none of the CTC-predictive use has been reliably demonstrated yet, there is the question of how to deal with the prognostic information offered by a regular CTC examination [46].

Patient's case (3): We enclose results of the postoperative CTC monitoring of a patient with TNBC (29 years old, stage I). CTC tests were provided during AT and subsequently in a follow-up period (**Figure 3**).

As shown in the figure, the presence of CTC with epithelial origin was detected during the AT course. Although the primary tumor was TNBC, CTC overexpressed oestrogen receptor (ESR) and HER2. Before the last docetaxel cycle (07/2015) during AT, we observed clustering of CTC and ER/HER2 lost. In the samples taken in 09/2015, 12/2015 and 09/2016 the number of CTC decreased and the characters of the cells changed from epithelial to mesenchymal (increased expression of VIM and ALDH). After the therapy completion, CTC remained resistant to antracyclines (epression of MRP1) for the rest of the time. We also registered elevated ERCC1 expression, which seems to be connected with stem cells like phenotype of CTC quite often as published in 2016 by Kasimir-Bauer et al. [47].

To be discussed: The persistence of the low amount of CTC with the signs of the stem cells and MRP1 resistant behaviour during the follow-up period is reported, but the patient is still in remission clinically.

Clinical implementation of CTC-examination: CTC monitoring after AT and during metastatic disease in a patient with HER2+ BC (Case report 4)

Hypothesis: CTC molecular characterisation during the metastatic disease follow up period may help to predict the therapy indication.

State of the art: CTC-positivity and HER2+ are both negative prognostic markers in BC. Together with HER2 discordance between the primary tumor and CTC (in studies 15%-35%), the disease becomes more aggressive and worse from the prognostic point of view [29, 48, 49]. The change of CTC phenotype is spontaneous [50] and behaviour of HER2+ CTC (proliferative potential) is different from those of HER2 negative (resistance to targeted therapy).

We observed the presence of CNS metastases in a patient (39 years old, stage II) with HER2+ locally advanced BC. CNS metastases were detected 11 months after the completion of trastuzumab therapy (**Figure 4**).

The patient started the NACT in 2014, the tumor responded to anthracyclines based therapy well but the effect of taxanes and trastuzumab was quite poor. CTC expressed HER2 at the beginning of the disease therapy, but not later during the taxane-based therapy.

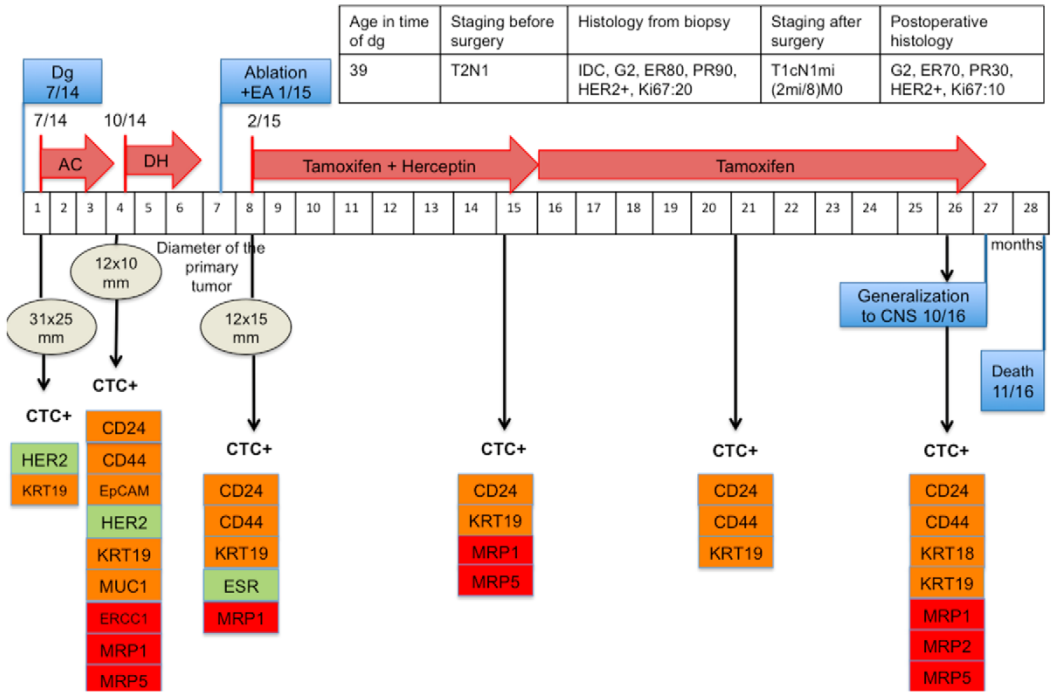


Figure 4. CTC monitoring in a patient with HER2- positive breast cancer. CTC positivity: CTC+, AC: doxorubicin + cyclophosphamide, DH: docetaxel + herceptin, EA: axilla exenteration, dg: diagnosis, ER: oestrogen receptor, PR: progesteron receptor, HER2: human epidermal growth factor receptor, G: grade, CNS: central nervous system, markers of stem cells: CD44/CD24, markers of epithelial cells: KRT18/19 (keratins), EpCAM (Epithelial Cell Adhesion Molecule), MUC1 (mucin), markers of chemoresistance: see **Table 2**.

During the AT (tamoxifen+ herceptin) CTC positivity was confirmed regularly. HER2+ CTC were found during AT with trastuzumab. Expression of ESR was detected in only two of CTC postoperative samples (02/2015 and 04/2015).

The elevation of CTC count and chemoresistance had been documented again before the disease progression and brain metastasis were detected. The expression of KRT18 and CD44 was elevated. Shortly after the trastuzumab therapy completion, CTC expressing HER2 were not present anymore. The patient's death occurred very quickly after the diagnosis of brain metastases.

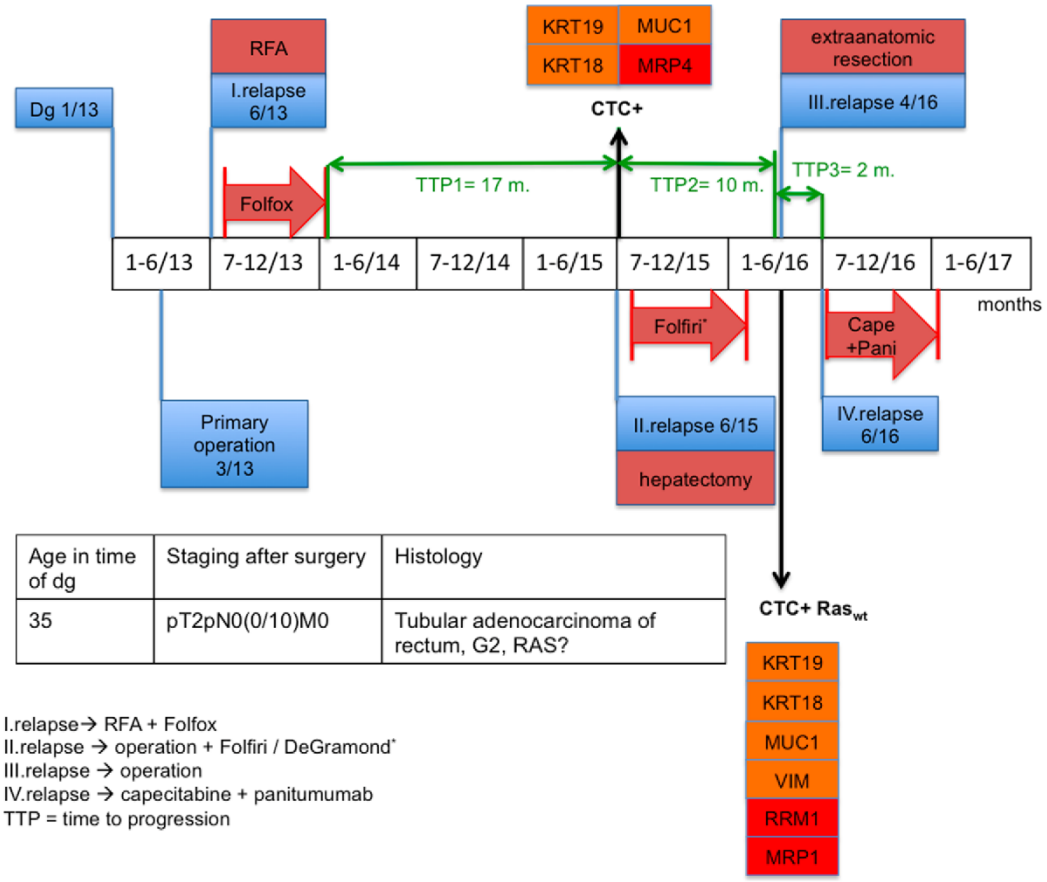
To be discussed: One could discuss the possibility of the re-administration of anti-HER2 therapy in the case of HER2+ CTC at the time of the brain metastases development. The effect of tamoxifen treatment could be redundant also as CTC did not express ESR. Such decisions do not reflect the existing recommendations and could be only used in clinical trials.

Clinical implementation of CTC-examination: CTC-testing after resection of metastases and early prediction of disease relapse in a patient with metastatic CRC

Hypothesis: CTC-examination including the chemoresistance profile analysis could help in the therapy indication in the metastatic disease course.

In patients with CRC and isolated metastatic liver disease, the presence of CTC has already been examined in several studies. The observation period included the time before and/or after the resection or radiofrequency ablation (RFA) [51-53].

According to the type of the detection method, CTC were found in 10-30% of patients before surgery, 29-50% of patients during the surgery and in 5-28% of patients after the surgery. The presence of CTC in the time during or after the surgery had a prognostic significance.



Age in time of dg	Staging after surgery	Histology
35	pT2pN0(0/10)M0	Tubular adenocarcinoma of rectum, G2, RAS?

I.relapse→ RFA + Folfox
II.relapse → operation + Folfiri / DeGramond*
III.relapse → operation
IV.relapse → capecitabine + panitumumab
TTP = time to progression

Figure 5. CTC-testing after resection of metastases in a patient with metastatic colorectal cancer. CTC positivity: CTC+, dg: diagnosis, G: grade, RAS: group of oncogenes, wt: wild type, RFA: radiofrequency ablation, m: months, cape: capecitabine, pani: panitumumab, FOLFOX/FOLFIRI: chemotherapy regimens (see main text), KRT18/19 (keratins), MUC1 (mucin), VIM (vimentin), genes of chemoresistance: see **Table 2**.

The monitoring of CTC in real-time and the observation of their dynamic behaviour would help to detect early the disease relapse. Properties of CTC could also help to predict an individual risk of disease relapse [54] and to choose the therapy after the resection of liver metastases. The benefit of the targeted therapy in the adjuvant indication has not been demonstrated in CRC yet [55-58]. The presence of CTC with RAS wild type (RAS_{wt}) properties could change this situation by positive selection of CRC patients.

Patient's case (5): The patient (35 years of age) with CRC was undergoing a surgery because of adenocarcinoma of rectum. The postoperative staging was T2N0(0/10)M0, the status of RAS could not be examined because of heavy DNA fragmentation. Short time after the surgery, liver metastasis developed in the left liver lobe. The tumor was cured by RFA and ACT (FOLFOX regimen) (**Figure 5**).

18 months later the second liver relapse appeared. Liver metastasis was resected again. The patient was secured with a systemic therapy (FOLFIRI) and blood collection for CTC examination was indicated. The results were showed CTC presence by molecular analysis, higher expression of keratins and MUC1 was confirmed, no mesenchymal markers were detected. A relatively small number (units of cells) of CTC could be the reason for quite a long time to the next progression (TTP2).

The third liver relapse developed after 10 months in 04/2016 and CTC test was positive again. Not only liver metastases but also CTC

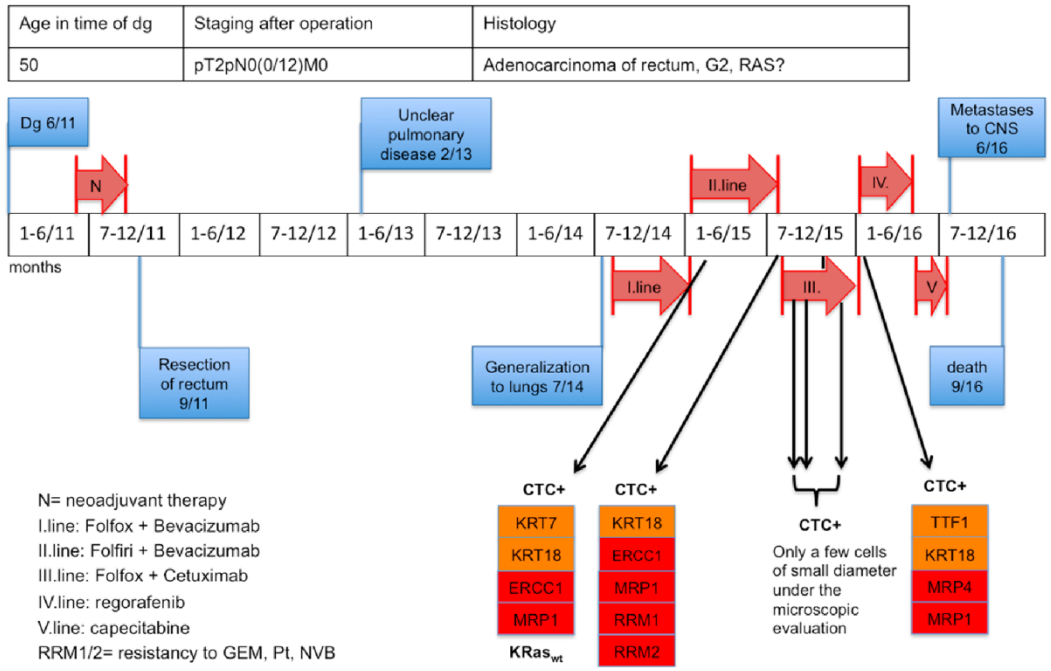


Figure 6. Assessment of KRAS mutation status from CTC in a patient with metastatic colorectal cancer. CTC positivity: CTC+, dg: diagnosis, G: grade, RAS: group of oncogenes, wt: wild type, CNS: central nervous system, FOLFOX/FOLFIRI: chemotherapy regimens (see main text), KRT18/19 (keratins), TTF1 (Thyroid transcription factor 1), genes of chemoresistance: see **Table 2**.

were tested for the presence of RAS mutations with a negative result (RAS_{wt} was confirmed). The expression of VIM, clustering of CTC and a high number of CTC were signs for the high disease relapse risk.

The 4th relapse developed in 2 months (TTP3). Because the patient refused any additional chemotherapy, an attempt was made by another liver resection, but with a short effect only. This patient was treated with anti-EGFR monoclonal antibody and capecitabine from 07/2016 to 02/2017.

To be discussed: The disease volume and KRAS status could be controlled by monitoring of CTC after the surgical removal of metastases.

Clinical implementation of CTC-examination: assessment of KRAS mutation status from CTC in patient with metastatic rectal adenocarcinoma

Hypothesis: CTC could present a relevant real-time information source displaying mutational status for genes relevant in the therapy indication process.

State of the art: As we know from the clinical trials, the wild form of KRAS oncogene ($KRAS_{wt}$) is associated with the sensitivity to anti-EGFR therapy, especially in the tumors of the left colon. According to some published studies, the discordance in the state of KRAS in comparison with the primary tumor and metastases is relatively small [59-61]. Likewise, relatively good correspondence in the state of KRAS between the primary tumor and peripheral blood is the reason for the effect of anti-EGFR therapy regardless the fact whether the result of KRAS is based on analysis of the primary tumor or CTC [62, 63].

The problem may occur in advanced lines of treatment because of previous therapy, which can cause super-selection of aggressive tumor clones. Discrepancies at various disease levels (primary tumor, metastasis, blood) could be striking [64]. Another possible benefit of RAS oncogenes or other genes determination from CTC is in cases, in which we lost the option to analyse RAS directly from the primary or secondary tumor.

Patient case (6): A case of a patient (50 years old, stage III) with rectal adenocarcinoma with unknown status of KRAS gene, because of low amount of primary tumor material, is presented (**Figure 6**).

The bulk in the left lung was discovered one year after the NACT therapy (02/2013). The patient has been under observation only because of bulk low diameter and the absence of other signs of an active disease. In 07/2014 lung metastases were confirmed by PET/CT and CTC were detected in the blood. The patient was treated with FOLFOX and bevacizumab in the 1st line and FOLFIRI and bevacizumab in the 2nd line but with only 6 and 3-month lasting effect. CTC examined after FOLFOX were resistant to oxaliplatin.

Before the initiation of the 3rd line of the therapy a biopsy from a newly discovered tumor mass in the liver was executed, but tumor cells were not aspirated. The analysis of KRAS was provided, based on CTC-material with the result of $KRAS_{wt}$. Nevertheless, combined FOLFOX and cetuximab therapy failed again. Only relatively small cells with several cancerous morphologic features were detected in the blood after the therapy completion. CTC expression profile was not done because of small amount of RNA.

We explain the therapeutic failure of the anti-EGFR therapy by tumor heterogeneity and by the administration of two previous therapy lines, which might cause the selection of chemoresistant cells subset (MRP1 and MRP4 expression).

The disease progressed macroscopically and new lesions in bones were discovered in 11/2015. We treated the patient with regorafenib and capecitabin in the next two lines but without any significant effect. The patient died in 9/2016 because of new CNS lesions. CNS metastases are not typical among CRC patients, and their presence explains the aggressiveness of the disease.

To be discussed: To influence the prognosis of the patient at the stage of generalization, the early treatment initiation is critical, but the verification of pulmonary focus (07/2014) could not be done, unfortunately. The liquid biopsy in such a case could replace screening, focused on the disease relapse verification. The molecular analysis of CTC including KRAS status analysis should be more perspective at the beginning of the disease. The effectiveness of anti-EGFR therapy was certainly affected by the previous treatment and by the chemoresistance of the disease, which was documented by examination of CTC. Increased EGFR expression could also be the cause of non-effect of regorafenib as a possible escape mechanism of the tumor cells in CRC patient [65]. The data correlating the status of RAS mutations are usually obtained from patients receiving the first line therapy [66, 67], however it was shown that the monitoring of CTC could be relevant in the advanced lines of therapy, too [68].

Clinical implementation of CTC-examination: strategy of using CTC for the palliative treatment guidance in a patient with NSCLC

Hypothesis: CTC-examinations could be used for EGFR mutation detection during the therapy course in a patient with NSCLC.

State of the art: The dynamic changes of CTC could reflect the prognosis of patients with NSCLC [69]. The chemotherapy efficiency decreases with the sequential selection of the chemoresistant tumor clones and its success can be a guarantee only by using drugs with the new mechanism of the effect, which could target on the slowly dividing cells and/or restoring the sensitivity of the tumor cells to cytostatics. The examination of CTC chemoresistance is one way of how to better choose the potentially effective cytostatic in palliative care. The mutational analysis of CTC may offer new information on EGFR-mutational status, identifying T790M mutation associated with anti-EGFR treatment resistance.

Patient's case (7): The case of 47-year old patient with stage IV NSCLC treated with combined carboplatin and pemetrexed therapy in the 1st line is presented. Her disease had the character of adenocarcinoma without mutations in genes EGFR, KRAS, NRAS, BRAF and ALK fusion was also not found in the primary tumor. The therapy was conducted from 01 to 05/2016. The examination in 02/2016 showed the presence of CTC with the expression of TA-associated genes EpCAM, MUC1, KRT18 and KRT19. CTC showed resistance to platinum

(ERCC1) and cross-resistance to several other antineoplastic agents (MRP1).

CT (computer tomography) scan from 05/2016 showed a mild non-effect of the therapy. The control blood test was carried out at the same time, the characterization of CTC changed partly, and the expression of VIM was newly verified. The disease developed more multi-resistant cells (expression of MRP1, MRP2, MRP4, MRP7, and ERCC1). Based on this result and based on the preserved sensitivity of the disease on derivatives of 5-fluorouracil (MRP5 expression has not been proven) and gemcitabine (expression level of RRM1 or RRM2 was not elevated), we indicated the treatment with gemcitabine and capecitabine in the 2nd line.

CT scan from 09/2016 showed a slight progression of a one pulmonary node but also the regression of tumors in other locations. An unresponsive focus was subsequently irradiated and after the completion of RT (09-10/2016) we continued with palliative treatment in the mentioned scheme till 12/2016. The control CT scan unfortunately revealed further bilateral progression of lung focuses. Despite of this result we declare the effect of the second-line treatment lasting for 6 months as successful.

To be discussed: CTC-assisted therapy supplemented by chemoresistance testing may contribute to a better therapeutic effect.

Clinical implementation of CTC-examination: typing of tumors with unknown primary site (C80) or duplicate tumors

Hypothesis: CTC-examination could be used for diagnostics of tumors with unknown origin or for the differential diagnosis in patients with duplicate tumors.

State of the art: CTC could be beneficial in tumors of the unknown primary site (diagnosed as C80). The inter-individual heterogeneity or the tumor dedifferentiation delimitate successful typing of known origin tumors as well as of C80 [70]. Detailed analysis of DNA allows to find deviations and mutations of genes involved to the pathogenesis of C80 [71], which could be the aims for the targeted therapy [72]. CTC represent a possible extension of the knowledge obtained from the tissue biopsies [73].

Patient's case (8): A 57-years old patient with duplicate CRC and PC, CTC examination was indicated to obtain prognostic information and to identify the type of CTC. CRC was resected; the post-operative stage was pT3pN0(14)M0, microsatellite stable (MSS). The PC stage T3b-N0-1, GS 3+4 was planned to examine by using choline- PET/CT. We discussed the need of AT in CRC. We considered both cancers as potentially aggressive; CRC because of their biological behaviour, PC because of the extent of the disease.

Detected CTC overexpressed following TA-genes: KRT18, KRT19, VIM, ALDH, VEGF, AMACR. The subset of genes confirmed presence of the cells with epithelial origin (keratins), but the elevation of stem cell markers (VIM, ALDH1) was also demonstrated. Additionally VEGF expression supporting the tumor angiogenesis was elevated. The cells were exhibiting morphological features of the cells found in the patients with CRC, but the elevated expression of the AMACR gene could be ascribed to the cells of prostate origin. We concluded that probably the both cell types from both tumor types were present in the patient's blood. The genes associated with the chemoresistance to anthracyclines (MRP1) and platinum (ERCC1) were detected.

We also indicated Oncotype DX Colon Cancer (Genomic Health, USA) examination with the result of the middle to high risk of the disease relapse according to the molecular print of the primary colon cancer (score of recurrence 39).

As RT of PC was planned, we recommended capecitabine as adjuvant monotherapy for CRC and dipherelin as the primary neoadjuvant treatment for prostate cancer.

To be discussed: The CTC examination helped us to distinguish the risk of relapse in two different malignant diseases. Stage II CRC does not always require ACT. The liquid biopsy could predict the need of the post-operative therapy in such cases. In comparison to molecular assays targeting the primary disease, the liquid biopsy offers a real-time monitoring of the CTC volume in time.

Discussion

The clinical evidence for the predictive value of CTC is still limited. Our two-step detection pro-

tocol combining a size-based filtration with the both cytomorphological and molecular characteristics of CTC may identify CTC in patient samples, where they cannot be detected by other methods (e.g. EpCAM-based separations). In the reported CTC positive samples (24/34 i.e. 70.6%) EpCAM expression has been confirmed in 2 samples (8%) only, expression of KRT 18/19 in 23 samples (95.8%) and MUC1 in 7 samples (29.2%). The changes in the number of CTC in responders compared to non-responders suggest that CTC properties are different in the patients with the same disease undergoing the same treatment.

The highest possibility for the CTC clinical implementation is in the palliative indication. The liquid biopsy- navigated therapy based on the detection of certain types of mutations in NSCLC patients is already part of a clinical care. We expect a similar use of CTC-testing in other diagnosis (e.g. the determination of RAS status in CRC or ARV7 in CRPC patients) and in the cases of the primary disease, where the tumor tissue is not approachable for a biopsy verification.

Considering the chances for CTC implementation in AT, monitoring of CTC in the patients in remission after the completion of the primary treatment is the only way how to actively intervene to the course of this period. It is evident that a disease relapse may occur even years after the primary diagnosis. The persistence of CTC in the blood of the patients in remission after primary treatment significantly increases the risk of a relapse. Long observational periods increase the already limited budgets for the cancer treatment. The liquid biopsy could be a promising inexpensive screening method (in the context of a comprehensive pharmacoeconomic assessment) [74].

To sum up, in all the presented case reports it has been shown that the aggressiveness of the disease may be defined by the persistent CTC long after the completion of the primary therapy (e.g. NACT, AT). Subsequently, possible therapeutic strategy of "watchful waiting" combined with the administration of systemic therapy apart from the completion of the primary treatment could be of a help.

Considering the results of the CTC-chemoresistance test (e.g. resistance to the anthracyclines defined by MRP1), the patients could overcome the "watchful waiting" periods with a support of an "additional" AT, e.g. by capecitabine administration after NACT in BC. Similarly, if low amount of CTC with signs of the stem cells in the follow up period is reported, the metronomic strategy of therapy could be considered.

Another situation has been described when HER2+ CTC were detected in the patients after the terminated anti HER2-therapy. One could discuss the possibility of a re-administration of the anti-HER2 therapy, especially at the time of the brain metastases development.

Our case reports show, monitoring of CTC, not only in BC but also in CRC and NSCLC, could control the disease volume after the surgical removal of metastases. In the last two mentioned cases, CTC could be used for molecular subtyping of the tumor, which is in CRC and NSCLC a necessary condition which allow the anti-EGFR treatment indication.

In all of the cases the CTC-assisted therapy, supplemented by the chemoresistance testing may contribute to a positive therapeutic effect. In comparison to the molecular assays targeting the primary disease, the liquid biopsy offers real-time monitoring of CTC volume in time.

Using CTC in the context of the disease diagnostic in tumors of unknown primary site or in the patients with a duplicate tumor could accelerate the therapeutic management of the cancer patients in general.

Based on the data presented, we assume, that the liquid biopsy could significantly improve the ability to monitor the malignant disease, to predict the treatment efficacy and to provide an additional base for the complementary use of CTC in concordance with conventional histology in the future.

Disclosure of conflict of interest

None.

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13.4. Publication IV

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Original Article

Molecular characterization of circulating tumor cells in ovarian cancer

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Abstract: The main focus of the study was to detect circulating tumor cells (CTCs) in ovarian cancer (OC) patients using a new methodological approach (MetaCell™) which is based on size-dependent separation of CTCs and subsequent cytomorphological evaluation. Cytomorphological evaluation using vital fluorescence microscopy approach enables to use the captured cells for further RNA/DNA analysis. The cytomorphological analysis is then completed by gene expression analysis (GEA). GEA showed that relative expression of EPCAM is elevated in CTC-enriched fractions in comparison to the whole peripheral blood sample and that the expression grows with *in vitro* cultivation time. Comparison of the relative gene expression level in the group of peripheral blood samples and CTC-fraction samples confirmed a statistically significant difference for the following genes ($p < 0.02$): KRT7, WT1, EPCAM, MUC16, MUC1, KRT18 and KRT19. Thus, we suggest that the combination of the above listed genes could confirm CTCs presence in OC patients with higher specificity than when GEA tests are performed for one marker only. The GEA revealed two separate clusters identifying patients with or without CTCs.

Keywords: CTCs, circulating tumor cells, ovarian cancer, cultivation, in vitro, MetaCell, gene expression

Introduction

Circulating tumor cells (CTCs) are tumor cells shed from primary and metastatic sites that circulate in the peripheral blood and can be detected by many advanced methods. The cells are present not only in patients with distant metastases, but also in patients with early, localized tumors. It is important to develop and optimize CTCs detection methods for future management of malignant diseases, especially to enable real-time monitoring of treatment efficacy and identification of new therapy targets and resistance mechanisms.

Recent studies have indicated the presence of a CTCs subpopulation that shows features of Epithelial-mesenchymal transition (EMT) in patients with epithelial origin tumors [1, 2] and the importance of detecting new markers which are not downregulated by the induction of EMT, enabling CTCs capture.

The main focus of the study was to detect CTCs in ovarian cancer (OC) patients using a new methodological approach which is based on size-dependent separation of CTCs and subsequent cytomorphological evaluation. Cytomorphological evaluation using vital fluorescence microscopy approach enables to use the captured cells for further RNA/DNA analysis. The cytomorphological analysis is then completed by gene expression analysis.

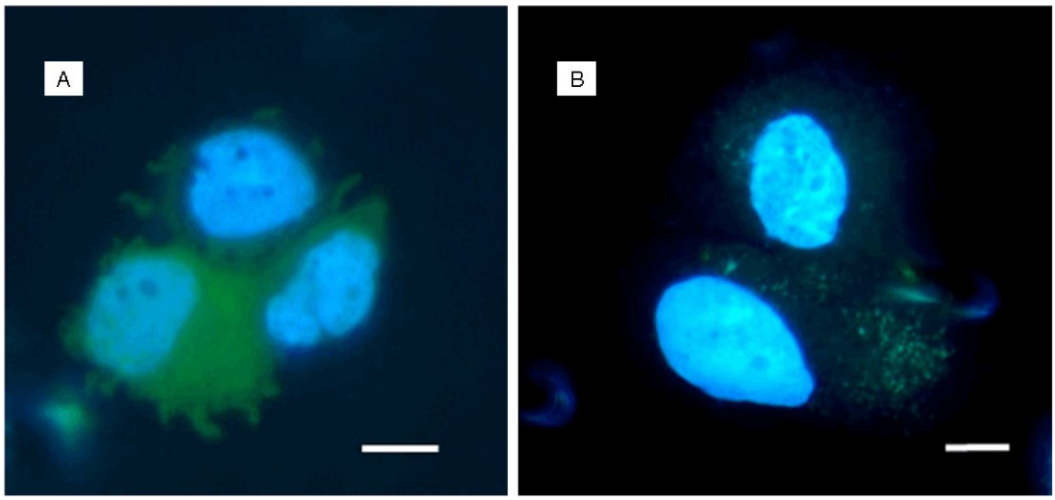


Figure 1. CTCs isolated from patients with ovarian carcinoma, captured on the separation membrane (vital fluorescent staining - NucBlue® and Celltracker®). Bar represents 10 µm.

Materials and methods

Patients

To date 40 patients with diagnosed OC have been enrolled in the gene expression study in accordance with the Declaration of Helsinki. All patients were candidates for surgery or surgical diagnostics. Based on informed consent, clinical data were collected from all participating patients. For each patient, approximately 8 mL of venous blood was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the blood draw.

CTCs enrichment and culture

A size-based separation method for viable CTCs enrichment from peripheral blood has recently been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) [3, 4]. The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (with pores of 8 µm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL with fluid. The standard 8 mL peripheral blood sample from patients suffering from OC was transferred into the filtration tube. Gradual transfer of the blood in several steps is preferred to prevent blood clotting on the membrane filter. The peripheral blood flow is supported by capillary action of the absorbent touching the membrane filter. The filtered CTCs were observed immediately after filtration on the membrane. The control and presence of filtered CTCs immediately after isolation eliminates false negative results of the examination. The membrane filter is kept in a plastic ring that is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and CTCs are cultured on the membrane in vitro under standard cell-culture conditions (37°C, 5% atmospheric CO_2) and observed by inverted microscope. The CTCs were grown in FBS-enriched RPMI medium (10%) for a minimum of 3-6 days on the membrane. Alternatively, the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide, or the separation membrane may be translocated on a microscopic slide. Microscopic slide is preferred if immunohistochemistry/immunofluorescence analysis is planned. If an intermediate CTCs-analysis is awaited, the CTCs-fraction is transferred in PBS (1.5 mL) to a cytospin slide. The slide is then dried for 24 hours and analyzed by histochemistry (May-Grünwald staining) and/or by automated immunohistochemistry protocols (Ventana, Benchmark Ultra, Roche) using standard differential diagnostic antibodies in the pathological evaluation process.

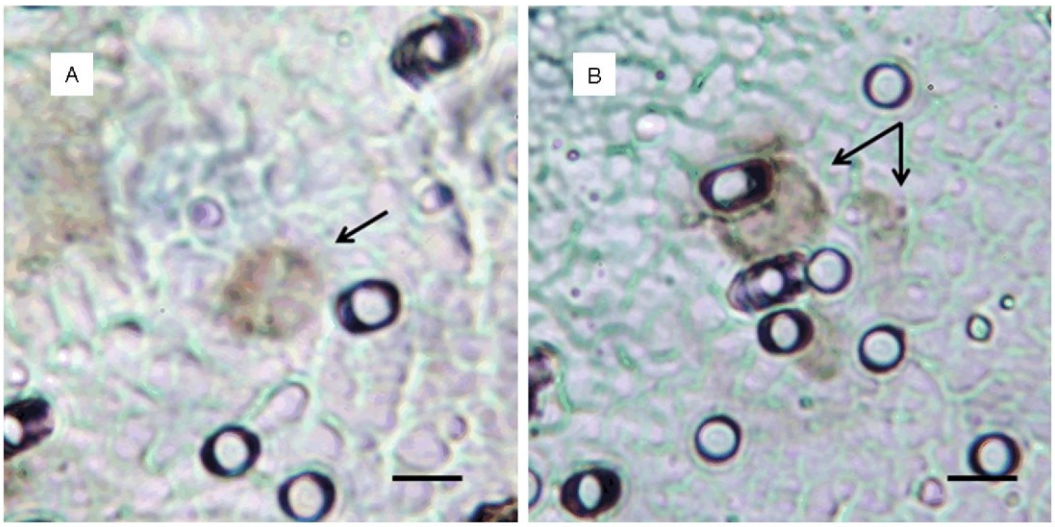


Figure 2. CTCs captured on the separation membrane after immunohistochemistry staining (WT-1 antibody) on Benchmark Ultra automat (Ventana, Roche). Bar represents 10 μm.

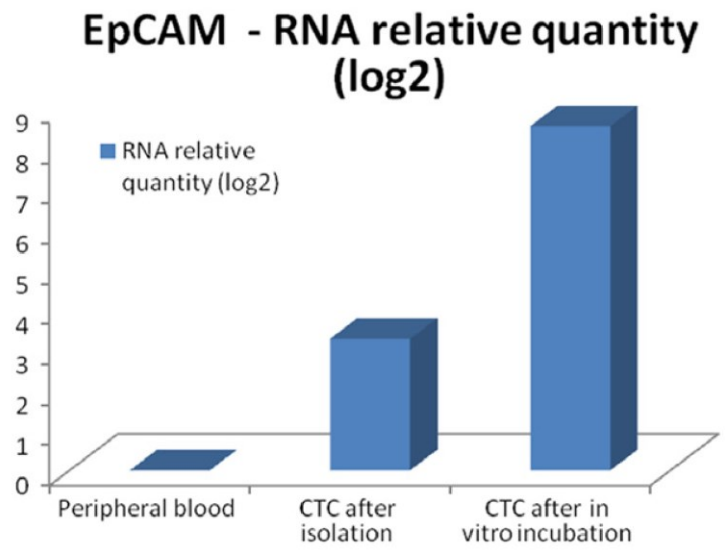


Figure 3. Relative expression of EPCAM RNA in peripheral blood and CTC fractions compared after qPCR analysis.

Cytomorphological analysis

The stained cells captured on the membrane were examined using fluorescence microscopy in two steps: (i) screening at x20 magnification to locate the cells; (ii) observation at x40/x60 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized, and the images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size ≥10 μm); (ii) irregular nuclear contour; (iii) visible cytoplasm, cells size over 15 μm; (iv) prominent nucleoli; (v) high nuclear-cytoplasmic ratio; (vi) proliferation, (vii) actively invading cells creating 2D or 3D cell groups.

Gene expression analysis (GEA)

The key purpose of GEA is to compare gene expression of tumor-associated markers in the CTC-enriched fractions to that in the whole blood (PK) and subsequently to confirm the origin of the cells on the separation membrane.

GEA allows up to 20 tumor-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostics markers for qPCR test are chosen in accordance with the suspected diagnosis.

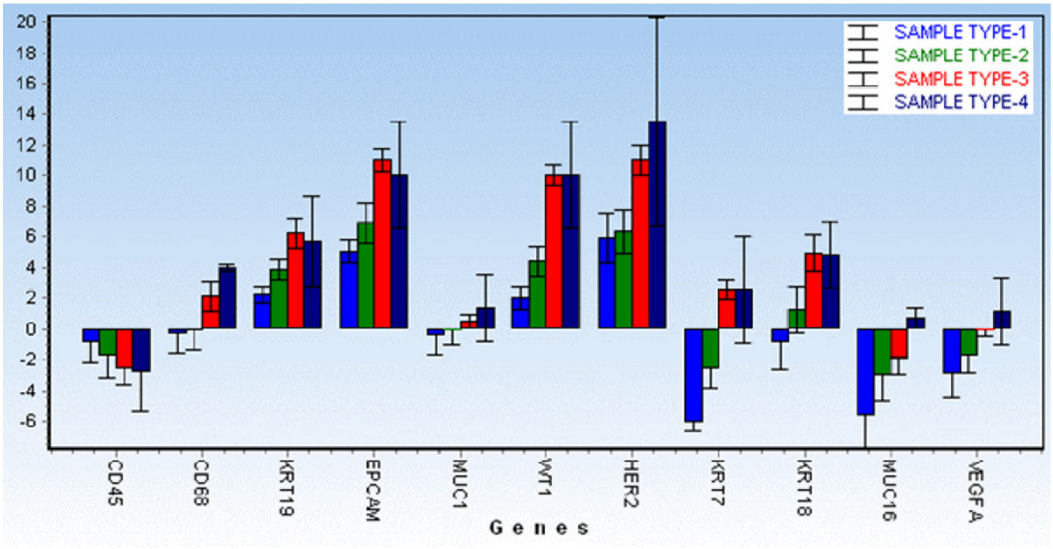


Figure 4. Comparison of averaged relative RNA expression for the genes, shown for all sample types (1-4). Sample type 1 (Peripheral Blood), Sample type 2 (CTC fraction stored immediately after separation process), Sample type 3 (CTC fraction after in vitro culture), Sample type 4 (bottom fraction - cells overgrowing the membrane).

RNA is isolated from the whole blood and CTC-enriched fraction on the membrane. There are two CTC-enriched fractions: the CTC enriched fraction of cells stored immediately after separation process (the so-called "virgin CTCs" - PK IZO) and/or the CTC enriched fraction of cells grown on the separation membrane *in vitro* (the so-called "membrane fraction" - PK SK). Some of the cells grown on the membrane *in vitro* may overgrow the membrane and set up a new cell culture on the culture-well bottom. These cells are analyzed as the "bottom fraction" (PK DK).

Finally, the CTC-gene expression analysis allows identification of the relative amount of tumor-associated markers in the whole blood and in CTC-enriched fractions. If the tumor-associated genes are highly expressed in the CTC fraction, a subsequent analysis of chemoresistance-associated genes is performed. Molecular analysis allows identification of which type of the chemotherapeutic agents may be of use in tumor therapy and assigned as personalized cancer therapy based on CTC.

The cells captured on the membrane are lysed by RLT-buffer with beta-mercapto-ethanol (Qiagen). RNA is then isolated using the RNeasy Mini Kit (Qiagen). The RNA from the whole blood is isolated with a modified procedure and the quality/concentration of RNA is measured by NanoDrop (ThermoScientific). As there are only a few hundred cells on the membrane, the median concentration of RNA is quite low (5-10 ng/µl). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA production. GEA was performed using Taqman chemistry with Taqman MGB-probes for all the tested genes (Life Technologies).

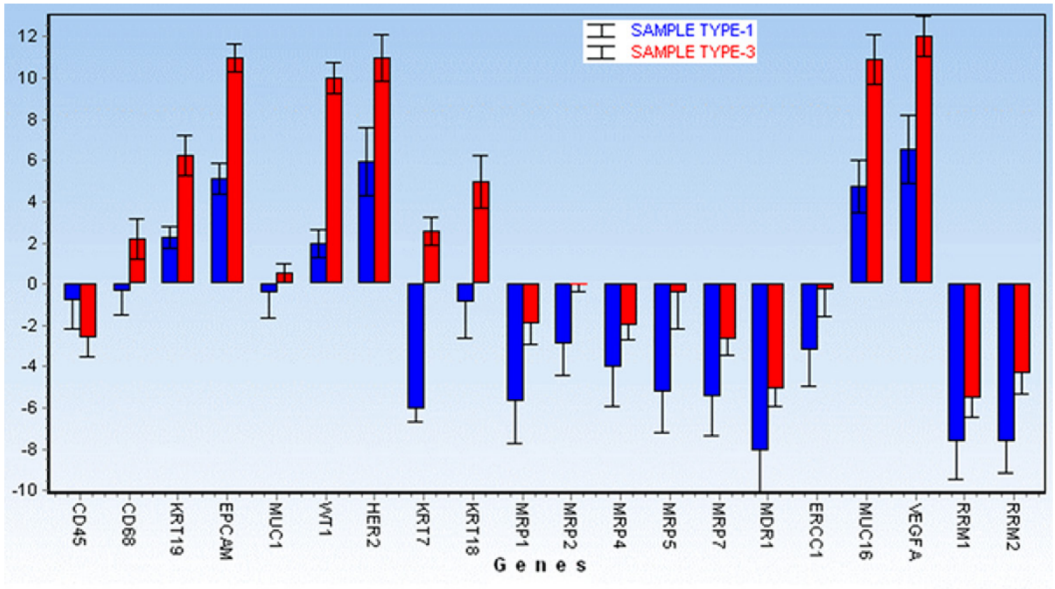
The following genes associated with tumorigenic character and therapeutic potential in ovarian cancer were chosen for the multimarker GEA panel: EPCAM, MUC1, MUC16, KRT18, KRT19, WT1, VEGFA, HER2. Additionally, genes associated with chemoresistance were tested (MRP1-10, MDR1, ERCC1, RRM1, RRM2).

Statistical analysis

All analyses were performed using clinicopathological information transformed into variables 0 and 1 if applicable for tested characteristics. Chi-squared test, t-tests, cluster analysis and-correlation analysis were outperformed using GeneX (MultiD, SE) and GraphPadPrism vs. 5 (Graphpad, US). *P*-value of less than 0.05 was considered as statistically significant.

Results

The main focus of the study was to detect CTCs in OC patients by a new methodological appro-



Gene	P
KRT7 (SAMPLE TYPE-3)	1.76E-06
WT1 (SAMPLE TYPE-3)	4.84E-06
EPCAM (SAMPLE TYPE-3)	1.49E-05
CD68 (SAMPLE TYPE-3)	0.000462
MUC16 (SAMPLE TYPE-3)	0.000525
KRT18 (SAMPLE TYPE-3)	0.000677
KRT19 (SAMPLE TYPE-3)	0.002262

Figure 5. Comparison of the relative gene expression level for the listed genes in the group of peripheral blood (Sample type 1) and CTC-fraction (after 3 days of in vitro culture - Sample type 3). Gene expression levels are relative to the whole peripheral blood data averaged for the patients group. A significant difference was noted for the following genes ($p < 0.02$): KRT7, WT1, EPCAM, CD68, MUC16, MUC1, KRT18 and KRT19. Thus, we suggest that the combination of the above listed genes should confirm CTCs presence in OC patients with higher specificity than when tests are performed for one marker only.

ach which is based on size-dependent separation of CTCs and subsequent cytomorphological evaluation. Cytomorphological evaluation using vital fluorescence microscopy approach (**Figure 1**) enables the use of the viable captured cells for further RNA/DNA analysis.

Patients diagnosed with different stages of ovarian cancer (OC) (n=56) were included in the study in 2014-2015. Cytomorphological analysis revealed that 58% were CTC-positive patients and 42% were CTC-negative patients. The results were confirmed by gene expression analysis with a slightly different ratio of CTC-positive and CTC-negative patients. The results of cytomorphological analysis were in agreement with multimarker gene expression analysis results in 92% of tested samples. The remaining 8-10% of "misdiagnosed patient samples" were re-analyzed by independent researchers.

There is a high potential hidden in the combination of cytomorphological and molecular analysis of CTCs in OC, especially due to the chemoresistance-gene analysis and automated immunohistochemistry protocols (**Figure 2**). Automation of immunohistochemistry staining could be a step needed for standardization of CTC-testing in clinical diagnostics.

Some patients were tested longitudinally so that we were able to analyze their CTCs throughout the whole year and demonstrate how CTCs evolve over time and during disease recurrence.

GEA showed that relative expression of EPCAM is elevated in CTC-enriched fractions in comparison to the whole peripheral blood sample and that the expression grows with *in vitro* cultivation time (**Figure 3**). Similarly, the increase in relative gene expression in the CTC-enriched fractions has been observed for KRT7, KRT18, MUC16 and WT1 in addition to EPCAM (**Figure 4**). Comparison of the relative gene expression level in the group of peripheral blood samples (Sample type 1) and CTC-fraction samples (3 days of *in vitro* culture - Sample type 3) confirmed a statistically significant difference for the following genes ($p < 0.02$): KRT7, WT1, EPCAM, MUC16, MUC1, KRT18 and KRT19

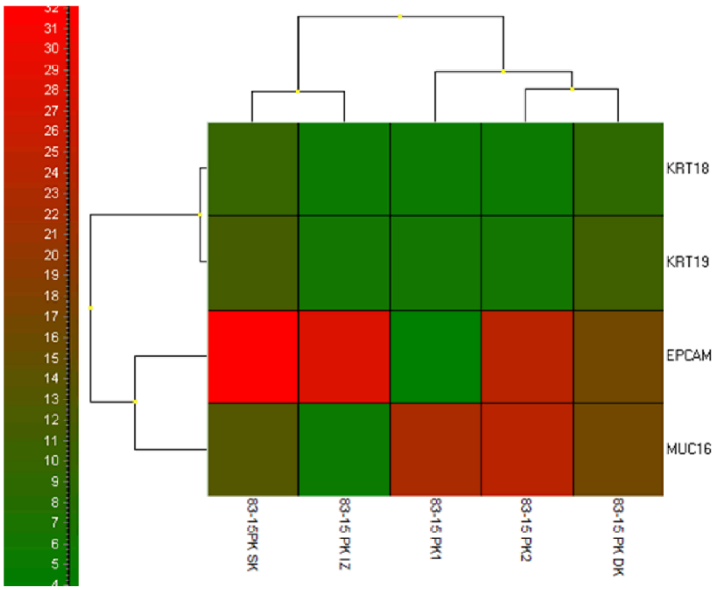


Figure 6. Cluster analysis for patient - RNA relative amount (log2) of tested genes in all of the tested fracions is shown in colours (please refer to the colour scale on the left). Tested fractions (peripheral blood PK), CTC after isolation (PK IZO), CTC after culture (PK SK), CTC overgrowing the membrane (PK DK). The highest expression of EPCAM was detected in PK SK fraction CTCs on the separation membrane.

(**Figure 5**). Thus, we suggest that the combination of the above listed genes should confirm CTCs presence in OC patients with higher specificity than when GEA tests are performed for one marker only.

If evaluated in an individual patient case, the GEA-cluster analysis shows that the highest EPCAM expression has been confirmed for the "membrane fraction" (sample type 3) (**Figure 6**). That is why all the "membrane fractions" (cells captured on the membrane and cultured *in vitro*) were compared together. The analysis revealed two separate clusters identifying patients with or without CTCs (**Figure 7**).

Discussion

The development of personalized treatment for patients with cancer depends on the specification of the molecular character of their disease. Therefore, there is a need to monitor tumor evolution and mechanism of treatment resistance. One of the approaches complementing traditional biopsy sampling could be the detection and analysis of CTCs.

The two most used enrichment techniques are the size-based filtration method and immuno-magnetic antigen-dependent method. The immuno-magnetic method is dependent on epithelial cell adhesion molecule (EpCAM), a tumor antigen highly expressed in epithelial cancers and at lower levels in normal epithelia [5]. EpCAM has always beenconsidered the ideal marker for the detection of CTCs in the peripheral blood of cancer patients.

The changes in CTCs phenotype are increasingly being recognized and it is clear that CTCs represent a heterogeneous entity. Indeed, a large amount of data demonstrates that in cancer the expression of epithelial surface markers might be transiently lost during the EMT process, to enable tumor cells to detach from the primary tumor and circulate into the bloodstream [6, 7].

Several lines of evidence have recently demonstrated that CTCs may adopt different strategies to protect themselves from the cell death fate, changing their phenotype from epithelial to mesenchymal, grouping into cell clusters or switching between the cancer stem cell state and the differentiated state of cancer cells [9]. Further studies widely demonstrated that EpCAM negative CTCs with mesenchymal cell like phenotype and downregulation of epithelial markers are frequently derived from EpCAM-positive primary tumors [10].

In contrast to the above, filtration methods are based on physical properties that allow size-based isolation of CTCs. The size-based method is an easy process which makes use of classic cytopathology evaluation criteria. Further gene-expression studies in CTCs are essential to determine tumor heterogeneity linking phenotypic differences. However, it is more difficult to preserve RNA than DNA, and the presence of wild-type DNA or RNA from WBC is a technical hurdle. As reported above,

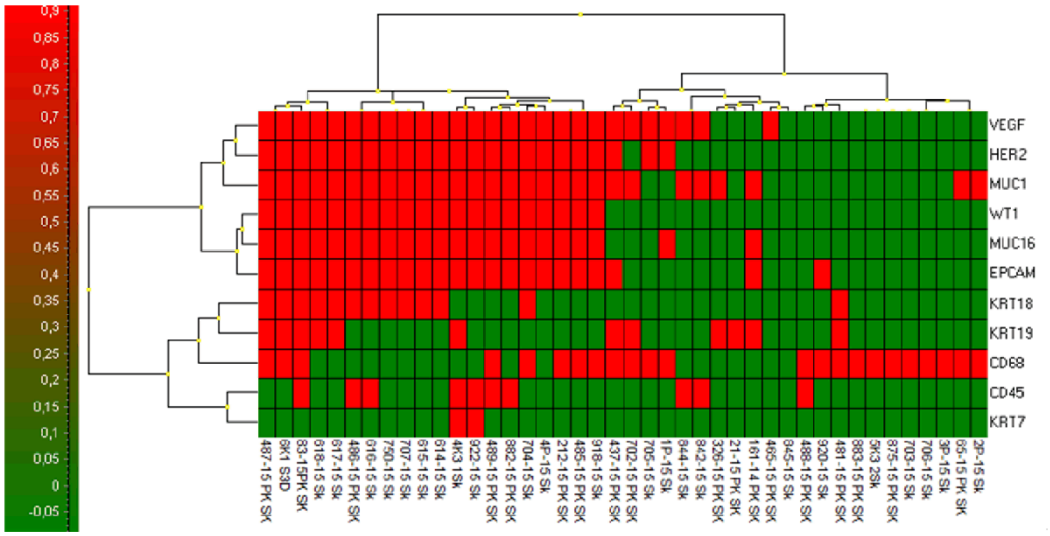


Figure 7. Cluster analysis of gene expression data RNA relative amount (log2) for CTC-membrane fractions (PK SK) of all tested patients clearly identifies CTC-positive and CTC-negative group of patients (Higher expression is shown in red).

the method of isolating viable CTCs followed by three days' incubation was used. The methodology protocol presented in this paper ensures a straightforward CTC-identification by combining cytomorphology, gene expression analysis and automated immunohistochemistry. It is very likely to be implemented into clinical routine, especially in the accredited pathology laboratories using automated immunohistochemistry staining.

GEA enables the researchers to detect changes in CTCs on a molecular level and thus to create an individual profile for each patient and to personalize the treatment.

Acknowledgements

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13.5. Publication V

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Original Article

The added value of circulating tumor cells examination in ovarian cancer staging

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Abstract: Delayed diagnosis of ovarian cancer (OC) is usually a cause of its high mortality. OC counts for one of the most aggressive gynecological malignancies. Noninvasive biomarkers may be used to help with diagnostic and treatment decisions in OC management. The incidence and clinical significance of occult OC cells (circulating tumor cells-CTCs) in the peripheral blood of patients with newly diagnosed or nondiagnosed OC at the time of surgical intervention were examined in our study. The objective of the study was to isolate and cultivate CTCs in OC patients (mainly stage IIIB-C) by a recently introduced size-based separation method (MetaCell®). CTCs were successfully isolated in patients with OC capturing cells with proliferation potential. The cells were enriched in good fitness, which enabled the short term *in vitro* culture of the CTCs. The CTCs may be used for further downstream applications (e.g. gene expression analysis) even if in the majority of the *in vitro* CTC cultures no confluence was reached. The CTCs were detected in 77 out of 118 patients (65.2%). CTC positivity was given to the relationship with different disease stage parameters with special focus on CA125 marker levels. The results show that the information on CTC presence may provide new and independent prognosis staging information to the patient description. Several interesting relationships of CA125, age and ascites presence are reported. As shown in our patient sample, patients with ascites tend to have higher CA125 levels, even if the CTCs were not found in the peripheral blood. It suggests that hematogenous dissemination is fully represented by the CTCs while lymphogenic dissemination is represented by elevated CA125. In this context, easy access to CTCs provided by the method applied in our study, both at the time of diagnosis and relapse, may become an increasingly valuable tool in future. This methodology may provide an opportunity for more personalized medicine where treatment for OC may be guided by information from an individual's CTC molecular profile.

Keywords: CTCs, circulating tumor cells, ovarian cancer, cultivation, in vitro, gene expression, CA125

Introduction

Ovarian cancer (OC) is the second most common malignancy of the female reproductive system in Western civilized countries [1]. Delayed diagnosis of (OC) is usually a cause of its high mortality. OC counts for one of the most aggressive gynecological malignancies. Only 25% of OC are diagnosed while the malignancy is still confined to the ovary, and the cure rate in these patients can reach 90%. If the disease spread already by the time of diagnosis, the probability of cure is really low [2]. For that reason, noninvasive biomarkers may be used to help with diagnostic and treatment decisions to ensure optimal patient management in OC.

Circulating tumor cells (CTCs) have demonstrated predictive and prognostic value in patients with metastatic breast, prostate, and colorectal cancers [3-5]. Results from prospective trials in each of these cancer modalities showed that

Table 1. Patient characteristics and CTC testing results

Patient characteristics	Total patients (N)	CTC positive (N)	%
	118	77	59
FIGO stage			
IA	5	4	80
IC	14	5	35.7
IIA	1	1	100
IIB	2	0	0
IIC	4	3	75
IIIA	3	1	33
IIIB	12	8	66.7
IIIC	69	44	63.8
IV	8	7	87.5
Grading			
1	1	0	0
2	19	10	53
3	52	35	67
NA	46	28	60.9
Histology			
Serous	81	55	62.4
Serous/borderline	4	1	25
Non serous (e.g. mucinous)	6	6	100
Clear cell type	3	1	33.3
Endometrioid	1	0	0
Undifferentiated	2	2	50
NA	21		9

patients with elevated CTCs at any time during the course of the treatment had a significantly shorter progression free survival (PFS) and overall survival (OS) than patients without CTCs in their peripheral blood [6-9]. Several studies have been conducted so far to assess CTCs in a similar context in OC. The results are summarized in Romero-Laorden review and suggest that CTCs and DTCs are associated with poor clinical outcomes in OC [10]. To implement CTCs examination as a prognostic tool in the clinical setting further multi-center clinical studies need to be conducted and at least two CTC-isolation methods need to be compared side by side to confirm their CTCs capture efficiency. Our study examined CTCs incidence and their association with clinicopathological characteristics of OC in patients with newly diagnosed or previously non-diagnosed OC at the time of surgical intervention. The objective of this study was to isolate and cultivate CTCs in OC patients by a recently introduced size-based separation method. The presence of CTCs was further evaluated in relation to CA125 values, the presence of ascites, age and other factors.

The focus was on the relation between CTCs and CA125. CA125 is the gold standard tumor marker in OC. Serum level of CA125 is used to monitor response to chemotherapy, relapse, and disease progression in OC patients. Given this, it seems reasonable to examine if CA125 may be used as a prognostic indicator in correlation to CTCs. CA125 level less than 35 U/mL is now considered normal [11, 12]. When stratified by disease stage, elevated levels were found in more than 90% of patients with advanced stage ovarian cancer but in only 50% of patients with stage I disease [15-17]. Most reports indicate that a rise in CA125 level precedes clinical detection by about 3 months [18]. Despite the limitations in the interpretation of a solitary CA125 value, this biomarker is widely used to prospectively evaluate therapeutic efficacy and monitor disease status among ovarian cancer patients [19, 20].

CA125 antigen is a serum marker which has been sufficiently well validated to be of use in routine clinical care [14]. We expect that based on further clinical studies, CTCs may become as important as CA125 in OC diagnosis and treatment? We hypothesized that CTCs and CA125 are independent factors in OC patients, adding new information values to OC staging. If this hypothesis is confirmed, CTCs may become a standard in OC management algorithm.

Materials and methods

Patients: To date 118 patients with diagnosed OC have been enrolled in the study in accordance with the Declaration of Helsinki. All patients were candidates for surgery or surgical diagnostics. Based on informed consent, clinical data were collected from all participating patients. The patient stage characteristics are shown in **Table 1** and **Figure 1**. For each patient, approximately 8 mL of venous blood was drawn from the antecubital veins and placed into S-Monovette tubes (Sarstedt AG & Co., Numbrecht, Germany) containing 1.6 mg EDTA/mL blood as an anticoagulant. The samples were processed at room temperature using an isolation procedure completed within 24 hours after the blood draw.

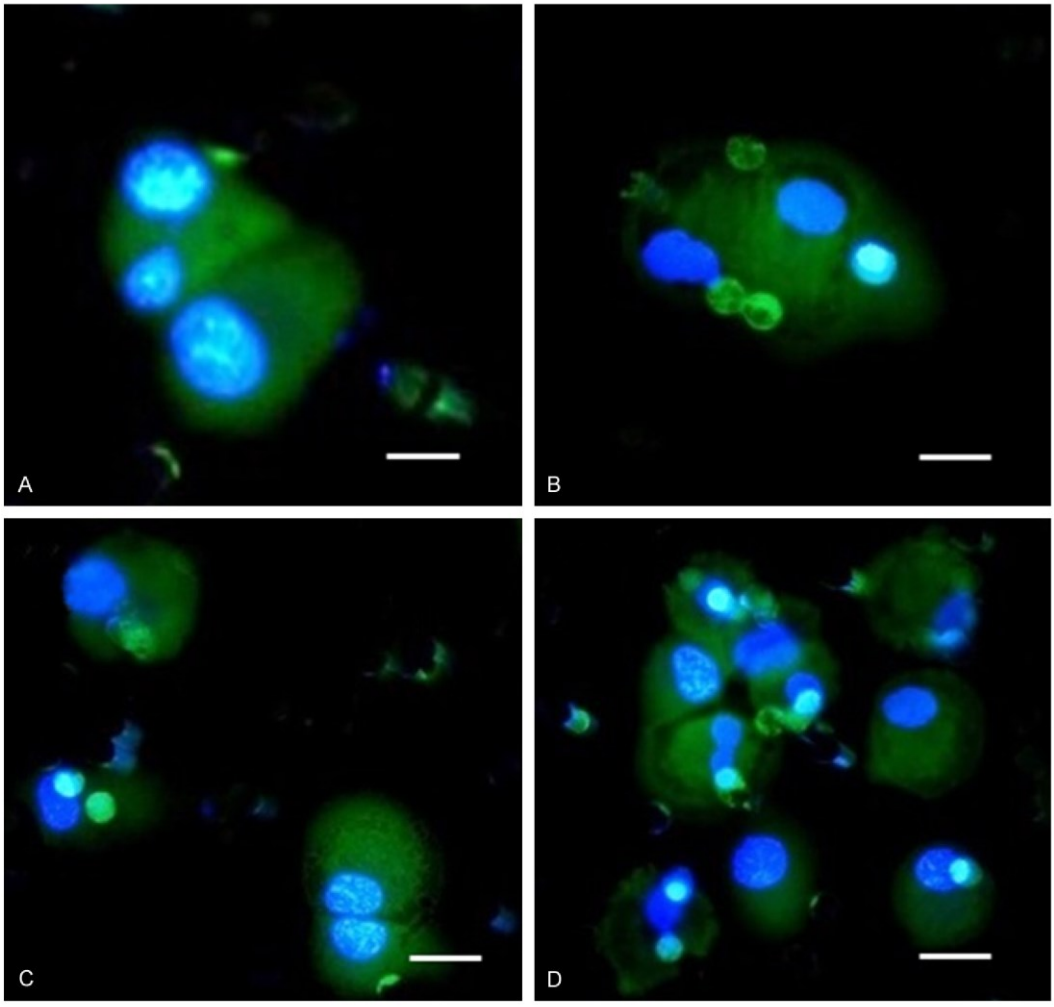

Figure 1. A-D. CTCs captured and cultured on the membrane filter stained by unspecific vital fluorescent nuclear (NucBlue™) and cytomplastic (Celltracker™) stain. The bar represents 10 µm.

CTCs enrichment and culture: A new size-based separation method for viable CTC enrichment from peripheral blood has recently been introduced (MetaCell®, MetaCell s.r.o., Ostrava, Czech Republic) [20, 21]. The size-based enrichment process is based on the filtration of peripheral blood through a porous polycarbonate membrane (with pores of 8 µm diameter). The minimum and maximum volume of the filtered peripheral blood may be adjusted up to 50 mL of fluid. The standard 8 mL peripheral blood sample from patients suffering from OC was transferred into the filtration tube. Gradual transfer of the blood in several steps is preferred to prevent blood clotting on the membrane filter. The peripheral blood flow is supported by capillary action of the absorbent touching the membrane filter. The filtered CTCs were observed immediately after filtration on the membrane. The control of CTCs presence immediately after isolation eliminates false negative results of the examination. The membrane filter is kept in a plastic ring that is transferred into the 6-well cultivation plate, 4 mL RPMI media is added to the filter top and CTCs are cultured on the membrane in vitro under standard cell-culture conditions (37°C, 5% atmospheric CO_2) and observed by inverted microscope (**Figure 3**). The CTCs were grown in FBS-enriched RPMI medium (10%) for a minimum of 3-14 days on the membrane. The cultured cells were analyzed by means of vital fluo-

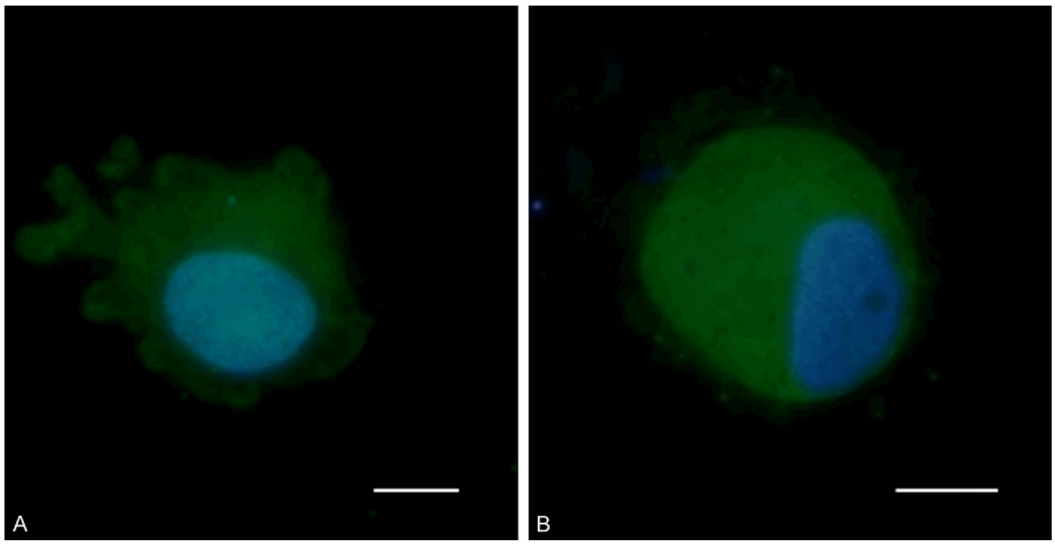

Figure 2. CTC captured and cultured on the membrane filter with visualized nucleus counterstained with vital nuclear (NucBlueTM) and cytoplasmatic (CelltrackerTM) stain. A. CTC isolated from a patient with diagnosed Clear Cell Ovarian Carcinoma. B. CTC isolated from a patient with Serous Ovarian Carcinoma. The bar represents 10 µm.

rescent microscopy using unspecific nuclear (NucBlueTM) and cytoplasmatic (CelltrackerTM) stain and by histochemistry (May-Grunwald staining).

Alternatively the enriched CTCs fraction can be transferred from the membrane and cultured directly on any plastic surface or a microscopic slide. Microscopic slide culture is preferred if immunohistochemistry/immunofluorescence analysis is planned. If an intermediate CTCs-analysis is awaited, the CTCs-fraction is transferred in PBS (1.5 mL) to a cytospin slide. The slide is then dried for 24 hours and analyzed immunohistochemically.

Cytomorphological analysis

The stained cells on the membrane (vital or fixed) were examined using light and fluorescence microscopy in two steps: (i) screening at ×20 magnification to locate the cells; (ii) observation at ×40 magnification for detailed cytomorphological analysis. Isolated cells and/or clusters of cells of interest (immunostained or not) were selected, digitized, and the images were then examined by an experienced researcher and/or pathologist. CTCs were defined as cells with the following characteristics: (i) with a nuclear size ≥10 µm); (ii) irregular nuclear contour; (iii) visible cytoplasm; (iv) prominent nucleoli. (v) high nuclear-cytoplasmic ratio, (vi) proliferating, (vii) growing in 3D-layers.

Gene expression analysis (GEA)

To confirm the origin of the cells on the separation membrane, CTC-gene expression analysis can be performed. Gene expression analysis (GEA) allows up to 20 tumor-associated markers in RNA from different cell fractions to be tested within a single quantitative polymerase chain reaction (qPCR) run. Differential diagnostics markers for qPCR test are chosen in concordance with the expected diagnosis.

The key purpose of GEA is to compare gene expression of tumor-associated markers in the CTC-enriched fractions to that in the whole blood.

Soon after, RNA is isolated from the whole blood and CTC-enriched fraction on the membrane. RNA can be isolated from the CTC fraction immediately after separation process (these are the so-called “virgin CTCs”) or/and from the CTC fraction grown on the separation membrane *in vitro* (the so-called “membrane fraction”). Some of the cells grown on the membrane *in vitro* may overgrow the membrane and set up a new cell culture on the culture-well bottom. These cells are analyzed as the “bottom fraction”.

Finally, the CTC-gene expression analysis allows identification of the relative amount of tumor-associated markers in the whole blood and in

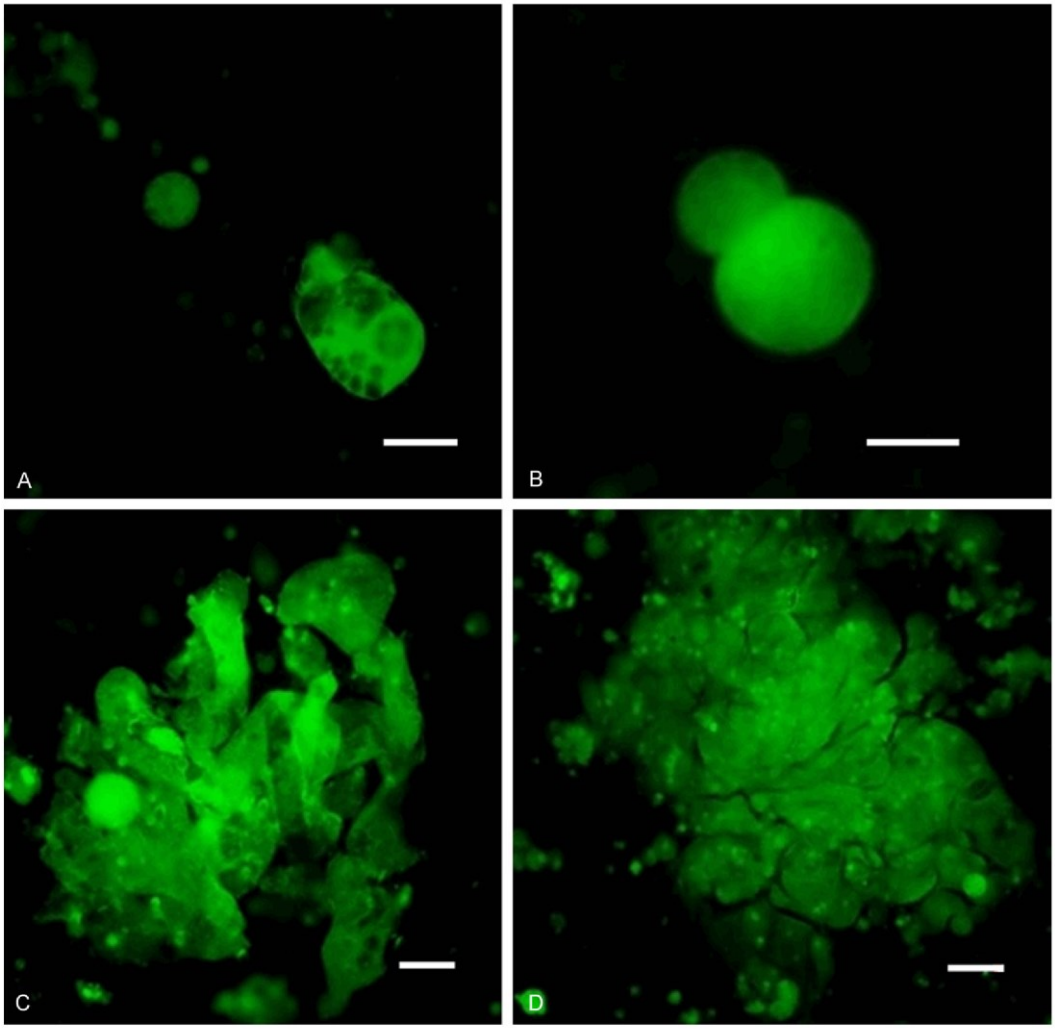

Figure 3. DTCs captured on the separating membrane shown immediately after enrichment (A, B) and after 6 days *in vitro* culture where cells already show differentiation towards epithelial character (C, D). Cells are visualized by Celltracker™. The bar represents 20 µm.

CTC-enriched fractions. If the tumor-associated genes are highly expressed in the CTC fraction, a subsequent analysis of chemoresistance-associated genes is performed. Molecular analysis allows identification of which type of the chemotherapeutic agents may be of use in tumor therapy and assigned as personalized cancer therapy based on CTCs.

The cells captured on the membrane are lysed by RLT-buffer with beta-mercapto-ethanol (Qiagen). RNA is then isolated using the RNeasy Mini Kit (Qiagen). The RNA from the whole blood is isolated with a modified procedure and the quality/concentration of RNA is measured by NanoDrop (ThermoScientific). As there are only a few hundred cells on the membrane, the median concentration of RNA is quite low (5-10 ng/µl). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for cDNA production. qPCR analysis was performed using Taqman chemistry with Taqman MGB-probes for all the tested genes (Life Technologies).

Statistical analysis

All analyses were performed using clinico-pathological information transformed into variables 0 and 1 if applicable for tested character-

Table 2. Clinicopathological characteristics and CTCs presence

		%	CTCs positive	%	CTCs negative	%	*P*-Value
CTC (N)	118						
CTC positive	73	61.8					
CTC negative	45	38.1					
CA125 (N)	85						
CA125 (1)	58	68.2	36	62.1	22	37.9	
CA125 (0)	27	31.8	14	51.9	13	48.1	0.3729
Ascites (N)	112						
Ascites (1)	53	47.3	39	73.6	14	26.4	
Ascites (0)	59	52.7	31	52.5	28	47.5	0.0216
Peritoneal carcinomatosis (N)	110						
Peritoneal carcinomatosis (1)	61	55.5	43	70.5	18	29.5	
Peritoneal carcinomatosis (0)	49	44.5	25	51	24	49	0.0367
Lymph node tested (N)	80						
Lymph node involvement (1)	23	28.7	16	69.6	7	30.4	
Lymph node involvement (0)	57	71.3	33	57.9	24	42.1	0.3322
Residual disease (N)	100						
Residual disease (1)	56	56	42	75	14	25	
Residual disease (0)	44	44	24	54.5	20	45.5	0.0321
Ascites (1) (N)	53						
Peritoneal carcinomatosis (1)	48	90.6	34	70.8	14	29.2	
Peritoneal carcinomatosis (0)	5	9.4	3	60	2	40	0.6156

Table 3. Correlation analysis of CTCs and other clinicopathological characteristics. Pearson correlation coefficients in the table are shown if reaching the level of significance ($p < 0.05$)

Pearson corellation test	CTC	Histology	Lymph node involv	Peritoneal Carcinomat	Ascites	Residual disease	new CA-125
CTC							
Histology							
Lymph node involvement							0.53
Peritoneal Carcinomatosis					0.7	0.74	0.71
Ascites	0.46			0.7		0.5206	0.43
Residual disease				0.74	0.522629		0.54
new CA-125			0.52	0.71	0.434698	0.54	

istics. The absolute CA125 levels were transformed into the categories normal (0) or elevated (1) to so called relative CA125 value. Chi-squared test, t-tests, cluster analysis, correlation analysis were outperformed using GeneX (MultiD, SE) and GraphPadPrism vs. 5 (Graphpad, US). *P*-value of lessn than 0.05 was considered as statistically significant.

Results

We report successful CTCs isolation in 77 out of 118 patients with OC (65.2%), capturing cells with proliferation potential in different histology OC subtypes. The cells enriched by size-based filtration remained in good fitness, unaffected by any antibodies or lysing solutions, which enabled theirs *in vitro* culture. The CTCs were cultured *in vitro* in short-term cultures (3-5 days) for further downstream applications.

CTCs in OC and their cytomorphological features

The size of the cells guided us in the process of cancer cell identification even without any additional staining (e.g. May-Grünwald-MGG). The standard staining method (MGG) has enabled

us to analyze the nuclei with nucleoli. Generally the nucleus was bigger than 10 μm itself and the cells did not present much of cytoplasm before *in vitro* culture. The nuclear-cytoplasmatic ratio is relatively high in cancer cells, but not in the *in vitro* cultured CTCs. The CTCs get big and elongated during in vitro growth, changing their nuclear-cytoplasmatic ratio. The cytoplasm of CTCs is relatively rigid if compared to the cytoplasm of DTCs (see **Figures 1**-**3**). The average cell size for the selected patients (n=10) was 23.4 μm (23.4 ± 6.7). Due to the cell size (15 μm), nucleus size (10 μm), shape, and nucleoli visualized by simple MGG-stain in the formerly fixed cells or by simple vital fluorescent staining procedure, CTCs may be identified simply by microscope. Cancer cells (CTCs/DTCs), captured on the separating membrane are shown on the **Figures 1**-**3**.

CTCs and molecular characterization

Gene expression analysis has been provided for some of tested patients (n=20). Preferably samples evaluated by cytomorphology as positive were included into gene expression profiling. CTCs in OC expressed MUC1, EPCAM in more than 90% cases, but MUC16 (reported as CA125) was detected only in 30% of CTCs. Additionally, KRT18 and KRT19 were heavily expressed in CTCs enriched and cultured fractions.

CTCs and clinicopathological criteria

The frequency of CTC positivity is summarized for different patient subgroups in **Tables 1** and **2**. As next CTC presence is put into correlation with other clinicopathological criteria in **Table 3**. Interestingly, under all the more serious disease conditions like higher FIGO stages, higher Grade, CTCs can be detected in more than 2/3 of the patients (more than 60% of tested patients are CTC-positive).

As shown in **Table 2**, CTCs were found in 30% of OC patients diagnosed with lymph node involvement. No significant correlation between CTC and LN-involvement has been reported in our OC group. 73.6% of the patients with ascites were CTC-positive. Up to 70% of OC patients with peritoneal carcinomatosis were CTC-positive. Patients with residual disease after surgery were also CTC-positive in 75%. Ascites was observed in 70% (48 patients) out of group of patient with peritoneal carcinomatosis data. As next, significance has been shown for correlation of CTCs and ascites, CTCs and peritoneal carcinomatosis, CTCs and residual disease.

In the context of CTC and CA125 testing the patients were divided into two subgroups based on the relative CA125 values. Only relative numbers for CA125 were taken into the analysis (elevated CA125=1, normal CA125=0). The results report that CTCs and CA125 behave like two independent biomarkers in the presented study. The independence has been confirmed by Chi-square test and correlation analysis (Pearson correlation coefficient) (**Table 3**). Pearson correlation coefficients for CTCs and other clinicopathological characteristics are shown only if reaching the level of significance ($P<0.05$). Another result of interest shown in the **Table 3** is the correlation of peritoneal carcinomatosis and residual disease with elevated CA125 values.

Additionally, cluster analysis of the tested clinicopathological parameters has been provided to demonstrate how different the group of OC patient undergoing surgery may be. Clearly there are 4 main clusters of the OC patients (see **Figure 4A**). There is a relatively big subgroup exhibiting no prognostically negative clinicopathological characteristic except of CTC-presence (3rd cluster from the left). Similarly, there is a cluster defined by peritoneal disease only (1st cluster from the left).

If as next variable CA125 (new CA125=relative CA125 value) is added to the cluster analysis, the subgroup of patients can be divided clearly into 2 distinct subclusters (**Figure 4B**). The first one is represented by elevated CA125 values, the second one is represented only by CTC presence. These facts may be used for better clinicopathological characterization of the OC patients, dividing them into the subgroups with hematogenous spread only and peritoneal spread only.

The presented data could support the hypothesis that the elevated CA125 levels are indicators of peritoneal OC spread in general. On the other site, CTC-presence is representing another type of metastatic spread-a hematogenous. As independence of CTC and CA125 has been shown (additionally on **Figures 5**-**7**), we may

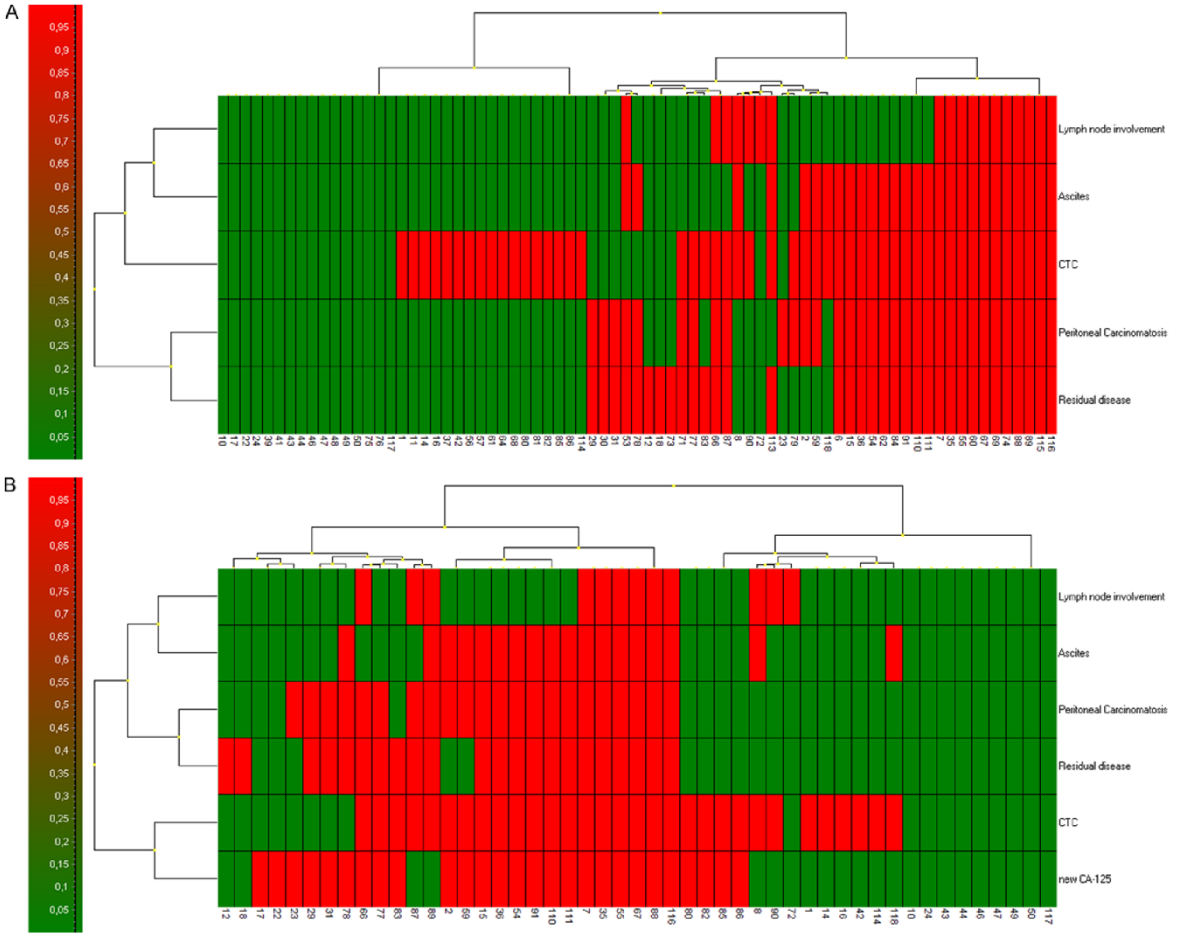
A
Lymph node involvement
Ascites
CTC
Peritoneal Carcinomatosis
Residual disease
B
Lymph node involvement
Ascites
Peritoneal Carcinomatosis
Residual disease
CTC
new CA-125

Figure 4. Cluster analysis of the clinicopathological criteria of OC-patients involved into this type f analysis. A. Cluster analysis without CA125 as variable. B. Cluster analysis including CA125 (elevation of CA125 is reported in relative numbers 1 (elevated) and 0 (normal)).

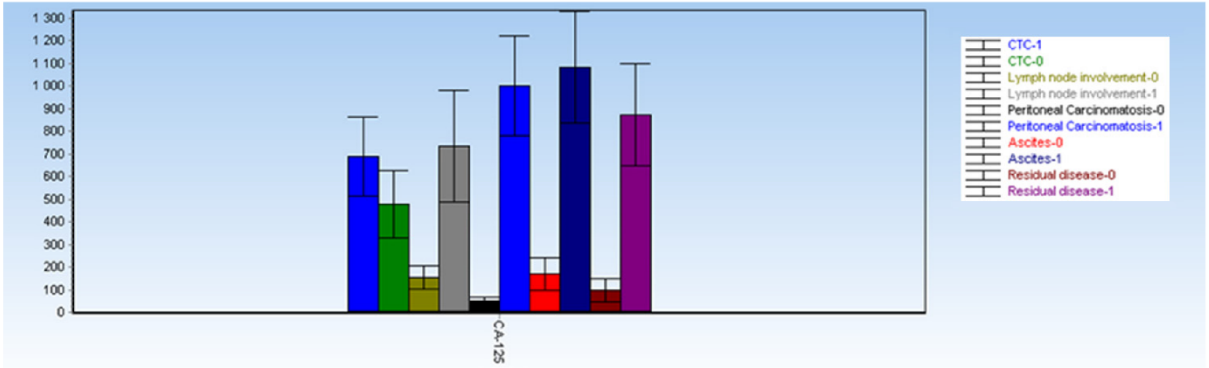

Figure 5. Means of absolute CA125 values (U/ml) are shown for different clinicopathological groups on the graph. The data show that the CA125 elevated levels are correlated to more serious disease characteristics (lymph node involvement, peritoneal carcinomatosis, ascites presence, residual disease presence), but not exclusively to the CTC-positivity. As shown CTC and CA125 are independent biomarkers in our group of OC patients.

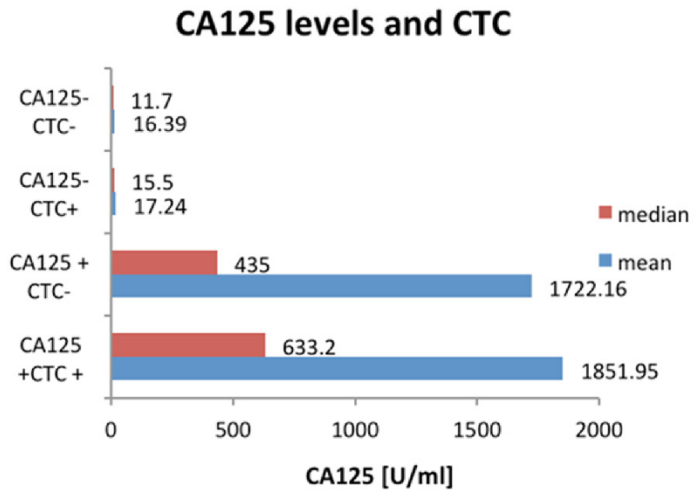

Figure 6. Absolute CA125 values (U/ml) in groups with CTC presence/absence and CA125-specification.

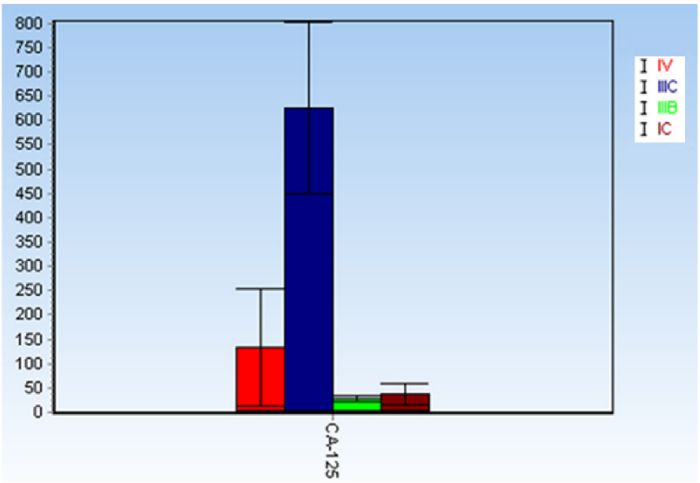

Figure 7. FIGO stages and absolute CA125 levels (U/ml). The data displayed on the graph show, that absolute CA125 levels do not correlate with FIGO stages. Elevated CA125 levels are typical to the FIGO IIIB stage (a blue column). FIGO IIIB is characterized by microscopic and macroscopic pelvic cancer mass presence. On the other site CA125 (U/ml) levels are associated with ascites presence ($P<0.05$). Taken together elevated CA125 could characterize peritoneal spread of the OC. CTCs were not seen more frequently at IIIB.

conclude that the both CTCs and CA125 tests may have its own role in the OC disease management.

Discussion

The theory of metastatic process is based on the fact that single tumor cells disseminate from the primary tumor either locally, leading to local metastases or through lymphatic vessels to regional lymph nodes or hematogenously to distant organs. Accurate diagnosis of disease stage can greatly influence the treatment modus in OC as well. It was believed that OC metastasizes via a passive mechanism by which ovarian cancer cells are shed from the primary tumor and carried by physiological movement and peritoneal fluid to the peritoneum and omentum. The discovery of hematogenous metastasis of OC via CTCs initiated rethinking of possible clinical implications of these findings for OCmanagement [23].

Patients with cancers that disseminate predominantly through the blood, such as breast, lung and prostate cancers, have substantially higher numbers of detectable CTCs [24]. So, it was surprising that the majority of patients (65.2%) investigated within planned surgical treatment in this study had detectable CTCs in their blood given that OC does not predominantly metastasize through the bloodstream.

Currently, no consensus on clinical utility has been reached for CTCs in OC patients. As reported in a review by Romero, more prospective validation and uniform methodology for CTCs detection is needed [10]. The CTC-examination may offer clinicians a more reliable method to identify disease aggressiveness earlier. The multicenter, randomized, exploratory study reported findings of patients with relapsed/recurrent advanced OC. Patients with baseline ≥2 CTCs had significantly shorter time to disease progression and shorter overall survival time than patients without CTCs in

their blood. This was independent of baseline CA125 value, platinum therapy status, tumor cell type and grade, prior taxane treatment, ECOG score, largest size and number of lesions, presence of ascites, race, and age. These data demonstrate that the prognostic value of CTCs is independent of established factors confirming their biological significance [25]. The independence of CTCs information on other clinicopathological characteristics (e.g. lymph node involvement and CA125 values) was shown by our study as well.

Prognostic significance has been confirmed for CTCs in several studies using different CTC-separation techniques [26-28]. Braun et al. observed a decreased overall survival as well as an increased rate of early, distant metastatic disease in patients with OC and DTCs detected in bone marrow biopsy [29].

Taken together CTCs may represent an opportunity to assess cancer spread directly and earlier than recent methods, which classify tumor growths in general. A functional methodology to harvest separated tumor cells from blood may provide researchers with a population of viable and proliferating cells to examine gene expression profiles or gene mutations in cancer.

Differences in isolation techniques and early disease stages make it difficult to report on recommendations resulting from presented OC-CTC studies. Nevertheless, the detection of CTCs is often hampered by the heterogeneity of the primary tumor and by the loss of epithelial antigens as occurs during epithelial to mesenchymal transition [30]. EpCAM (epithelial cell adhesion molecule) is definitely not a perfect marker for CTCs detection due to the high variation in its gene expression between tumor subtypes and its illegitimate transcription from leukocytes [31]. Furthermore cytokeratins, generic markers for epithelial CTCs, are also found on healthy epithelial cells released into circulation as well as on a subpopulation of granulocytes [32, 33]. For this reason, testing of other tumor-associated genes has been introduced into the CTCs evaluation process through our study. We were able to identify expression of tumor-associated genes on captured CTCs and confirm cancer origin of the captured cells by gene expression analysis.

As a result, even if the captured enriched CTC-population is contaminated by white blood cells, these can be eliminated by short-term *in vitro* culture. If still some white blood cells survive the short term *in vitro* culture, they may be identified by cytomorphological and gene expression analysis. Similarly, higher expression of tumor-associated genes can be detected if more cancerous cells are present in the enriched cell fraction used for testing.

Interestingly, under all the more serious disease conditions like higher FIGO stages, higher Grade, CTCs were detected in more than 2/3 of the patients (more than 60% of tested patients were CTC-positive) in our study.

Our study results suggest that MetaCell® techniques may be used to capture, identify and enumerate CTCs in patients with OC. This may provide useful additional and independent prognostic information independently on standard CA125 biomarker. As concluded from our data elevated CA125 could characterize peritoneal spread of the OC. Then CTCs could be a sign of hematogenous spread solely. From that context there is an extremely interesting group of patients who do not display CA125 elevation and are CTC positive.

The ability to isolate CTCs at an early disease stage and independently from epithelial antigens is an important improvement. The treatment of OC is increasingly likely to evolve into a more individualized approach, based on a better understanding of the molecular composition of each patient's tumor. In this context, easy access to CTCs, both at the time of diagnosis and relapse, should be an increasingly valuable tool in future years.

Our methodological approach may provide an opportunity for more personalized medicine where treatment for OC may be guided by information from an individual's CTCs molecular profile.

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Disclosure of conflict of interest

None.

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